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The Improvement of LC-MS/MS Proteomic Detection with Biomimetic Affinity Fractionation

Rong-Xiu Li, Qing-Qiao Tan and De-Xian Dong
Key Laboratory of MOE for Microbial metabolism and
College of Life Science and Biotechnology, Shanghai Jiao Tong University,
800 Dong-chuan Road, Shanghai 200241
China

1. Introduction

The systematic study of all proteins, referred to as proteomics, is performed to identify the components of a particular proteome and analyze global changes in protein expression in response to different stimuli. Over the past two decades, mass spectrometry has become an important tool for the analysis of proteins (Aebersold and Mann, 2003; Yates, 2004). It is estimated that a cell contains at least 10 000-30 000 different proteins that span a wide range of sizes, relative abundance, acidity/basicity, and hydrophobicity (Badock et al., 2001). As is known, the low-abundance and high-abundance proteins differ in concentration over nine orders of magnitude (Omenn, 2004), which results in the high-abundance proteins impeding the investigation of middle- or low-abundance proteins. The separation of the protein mixture into organelles or other multi-protein complex fractions prior to a proteomics analysis is usually the first step to increase the probability of detecting low-copy-number proteins (Rabilloud et al., 1998; Taylor et al., 2000; Cronshaw et al., 2002; Jiang et al., 2004; Gao et al., 2005). Efficient and highly resolving separation of the protein mixture into organelles or other multi-protein complex fractions prior to a mass spectrometry analysis is usually used as reducing the complexity of samples, which highly increases the probability of detecting low-copy-number proteins (Rabilloud et al., 1998; Taylor et al., 2000; Cronshaw et al., 2002; Jiang et al., 2004; Gao et al., 2005). Before the widespread use of shotgun methods, proteins were fractionated by 2D gel electrophoresis (2D-gel), a method that separates them according to pI and molecular weight (Harry et al., 2000; Gygi, et al., 2000; Motoyama & Yates, 2008). Although 2D-gel has excellent resolving power for intact proteins, the process is difficult to automate, laborious, and suffers from low throughput (Klose & Kobalz, 1995). The more fundamental drawbacks are the limited dynamic range and the exclusion of certain classes, such as low-abundance proteins (Gygi et al., 2000), integral membrane proteins (Braun et al., 2007), and proteins with extremes in isoelectric point (pI) and molecular weight (MW) (Corthals et al., 2000; Oh-Ishi et al., 2000).

Another explored proteins pre-fractionation methods is multidimensional liquid chromatography technique combined ion-exchange chromatography and/or hydrophobic chromatography and/or reverse-phase chromatography, which has been a powerful

technique to simplify the proteins in sample to 2D-gel in the proteomics research(Shen et al., 2004; Bergh et al., 2003; Jacobs et al., 2004).

One current method for the analysis of protein mixtures is proteolytic digestion followed by LC/MS/MS, often referred to as "shotgun" strategy. It has been proved powerful (Washburn et al., 2001; Wu et al., 2003; Shen et al., 2004). The heart of the method is the use of MS/MS to identify proteolytic peptides. However, it results in a complexity of millions of trypsin digest peptides, in which the signal of peptides of high-abundance proteins would mask the signal of middle- or low-abundance proteins. To simplify the sample and increase the detection probability of middle- and low-abundance proteins, suitable high abundance protein depletion and prefractionation methods were often adopted prior to trypsin digestion (Bergh et al., 2003; Jacobs et al., 2004; Gao et al., 2006; Liu and Zhang, 2007; Gao et al., 2008).

Affinity chromatography (AC) can be also used to reduce the complexity of the protein/peptide by selecting the specific target protein/peptide (Gygi et al., 1999; Pieper et al., 2003; Greenough et al., 2004, Tan et al.,2009a,2009b & 2010). Traditional affinity adsorbents comprise natural biological ligands, which are expensive to produce, show low binding capacities, have limited life cycles, and low scale-up potential. As a new affinity fraction, biomimetic chromatography uses synthetic affinity ligands for matrix, which could mimic the properties of natural ligands and circumvent these drawbacks of natural ligands by imparting resistance to chemical and biochemical degradation, displaying ease and low cost of production, and withstanding harsh sterilization without loss of performance (Lowe, 2001). It has been verified that many specific synthetic affinity ligands could be designed to purify different single proteins in our lab and other labs (Li et al., 1998; Teng et al., 1999; Gupta and Lowe, 2004; Melissis et al., 2007; Xin et al., 2007; Dong et al., 2008a).

Our lab has constructed affinity ligand library (thousands of ligands), and different ligands showed different absorbance effect to proteins such as purification, depletion of high abundance proteins, and enrichment of low abundance proteins (Dong et al, 2008b), which gives a imaginable application prospect in proteomics. In this Chapter, from biomimetic affinity ligands library in our lab, we first screened out some affinity ligands showing large absorption differences in band distribution, then, selected the affinity ligands having medium absorption ability; and finally a cascade and a tandem composition of these affinity ligands were contructed and applied in prefractionation of complex tissue proteins. Fractionated samples were analyzed by LTQ-MS/MS. After reducing the complicity of tissue protein sample, more protein information was able to be obtained from LTQ-MS /MS analysis. The cascade and tandem affinity biomimetic affinity chromatography exhibits wide application prospect in proteomics because of its low price, simplicity, high throughput, specificity of ligand absorbance and repeatability.

2. Materials and methods

2.1.1 Materials

Adult male Sprague-Dawley rats were purchased from Shanghai Laboratory Animal Center (Jiu-Ting, Shanghai, China). Sequencing grade modified trypsin was from Promega Corporation. The water used was Milli-Q grade (Millipore, Bedford, MA). Phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), guanidine hydrochloride, formic acid, acetonitrile, trifluoroacetic acid, and iodoacetamide were obtained from Sigma (St. Louis, MO). SDS, acrylamide and N,N'methylen-bis-acrylamide were purchased from

Amersham Pharmacia company. Unless stated otherwise, all reagents and chemicals were of the highest purity available.

2.1.2 Sample preparation

The rat liver tissue was cut into small pieces and cleaned with cold physiological saline solution (0.9% NaCl) to remove blood and some possible contaminants. Then, 5 g of tissue debris was rapidly mixed with 50ml pH 7.6 Tris-HCl lysis buffer, containing 1 mM PMSF, 5mM MgCl, 250mM Sucrose, 1 mM DTT. Tissue sample was homogenized in an ice bath, then sonicated for 800 s with the JY92-II Ultrasonic Cell Disrupter (Ningbo Haishu Kesheng Ultrasonic Equipments Co.,Ltd, China) at 50% of duty cycle and 450W output. After being centrifuged for 15 min at 150 000g, the supernatant was collected (cytosol fraction). Protein concentration of the sample was determined by the modified Bradford method described by Qu et al(1997), using bovine serum albumin (BSA) as standard.

2.1.3 Synthesis of affinity ligands

The affinity ligands library was constructed according to the methods of our lab(Dong et al 2008a). Briefly, Sepharose 4B was first activated with epichlorohydrin, then cyanuric chloride was coupled as a large spacer, and substitution of chloride in spacer with different amino compounds provides lots of ligands which have different sizes, shapes, and functionalities.

2.1.4 Evaluating of synthetic affinity ligands

Different gel-immobilized synthetic ligands (1ml) were respectively packed into polystyrene columns with porous discs at both the bottom and the top of the gel. The column was equilibrated with a 10-fold column volume of 10mM phosphate buffered saline (pH7.0). 4mg Cytosol fraction of rat liver protein was loaded onto the column, and left bound for 30 minutes. 10mM phosphate buffered saline (pH7.0) was applied to flush the column until the baseline of UV monitor (A280) went down to the bottom, and the flowthrough was collected. Then, 20mM Glycine-NaOH buffer (pH12.0) was used to elute out the bound proteins. Finally, 0.1M NaOH plus 30% ethanol was applied to wash and clean the column until the baseline of UV monitor went down to the bottom. All the elution fractions collected were used for subsequent SDS–PAGE electrophoresis. After comparison with absorbance protein profiles, selecting the ligands having large difference in absorbance characterization.

2.1.5 Trypsin digestion of each protein mixture fraction

Appropriate volumes of protein sample for each fraction were desalted and concentrated as above, lyophilized to dryness. 30 μg of lyophilized protein sample was redissolved in reducing solution (6M guanidine hydrochloride, 50mM Tris-HCl, 3mM DTT, pH8, 30 μl). The mixture was incubated at 60°C for 1 h and then 1.5 μl of 1 M iodoacetamide was added and incubated for an additional 30 min at room temperature in darkness. After denaturation, allow the reaction to cool and add 270 μl of 50mM ammonium bicarbonate buffer(pH 8.5). Then, modified trypsin was added to a final proteinase: protein ratio of 1:30(W/W), and the mixture was incubated at 37 °C overnight. The peptide mixture was lyophilized to dryness.

2.1.6 LTQ-MS/MS analysis

Redissolved peptides mixture was injected onto a Zorbax 300 SB-C18 peptide traps (Agilent Technologies, Wilmington, DE) to desalt, and separation was performed on a Zorbax 300SB-C18 reverse phase capillary column (300 μm inner diameter × 15 cm, Agilent Technologies) at (250 nl/min) with a linear gradient of 4-50% B over 50 min (A: 0.1% formic acid; B: 84% CH3CN and 0.1% formic acid), a step up to 100% B in 4 min, and then holding at 100% B for 10 min. The peak was online injected into a Finnigan LTQ (single linear quadrupole ion trap) mass spectrometer for peptide identification.

Mass spectrometry was on a Finnigan LTQ linear ion trap. The MS method consisted of a cycle combining one full MS scan with three MS/MS events (25% collision energy) followed by an MS3 event (35% collision energy). Dynamic exclusion duration was set to 30 s. The MS/MS and MS3 spectra from all the runs were searched using BIOWORKS protein identification software against database of ipi Rat. The SEQUEST filter was set to Charge +1,Xcorr≥1.9; Charge +2, Xcorr≥2.2; Charge +3, Xcorr≥3.75; and DelCN≥0.1。

2.1.7 Bioinformatics annotation tools

The theoretical pI and molecular mass values of proteins were from ExPASy Proteomics Server (http://cn.expasy.org/tools/pi_tool.html). The GRAVY values (http://www.bioinformatics.org/sms2/protein_gravy.html)were determined according to Kyte & Doolittle (1982). The TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001) was used to predict protein TM domains.

3. Prefractionation of rat liver cytosol proteins using tandem biomimetic affinity chromatography

3.1 Tandem mimetic affinity prefractionation

According to absorbance protein profiles (SDS-PAGE), we selected 5 affinity ligand columns having large band difference, and tandem combination of 5 columns could be applied as prefractionation of tissue lysis prior to LTQ shot –gun LC-MS proteome analysis. A schematic illustration of the workflow is shown in Fig. 1. 4mg Cytosol fraction of rat liver protein was loaded into the tandem columns. After rat liver proteins being coupled for 30 minutes, 10mM phosphate buffered saline (pH7.0) was applied to flush the tandem columns until the baseline of UV monitor (A280) went down to the bottom. Then, 5 tandem columns were divided into single column, and bound protein of each column was respectively eluted by 2-fold column volume of 20mM Glycine-NaOH buffer (pH12.0). 5 elution fractions were instantly adjusted into pH 7-8. After being desalted and concentrated using Microcon ultrafiltration membranes (3,000 Nominal Molecular Weight Limit, Millipore), all the elution fractions collected were used for subsequent SDS–PAGE electrophoresis and LTQ analysis.

3.2 Evaluating for differential protein absorbance profiles of mimetic affinity ligands

After analyzing the screening datas of our lab in serum and leech proteins, we selected the ligands showing medium absorbance ability. 60 ligands were selected from affinity ligands library of our lab, and the absorbance characterization of these ligands was reevaluated using rat liver cytosol proteins as materials. From the SDS-PAGE profiles of each ligand, we found that some ligands showed large absorbance differences in band distribution to rat liver cytosol proteins (FIG. 2). Partial examples of proteins absorbance profiles were shown in FIG. 2, and each ligand showed its own specific binding ability to some proteins, but low

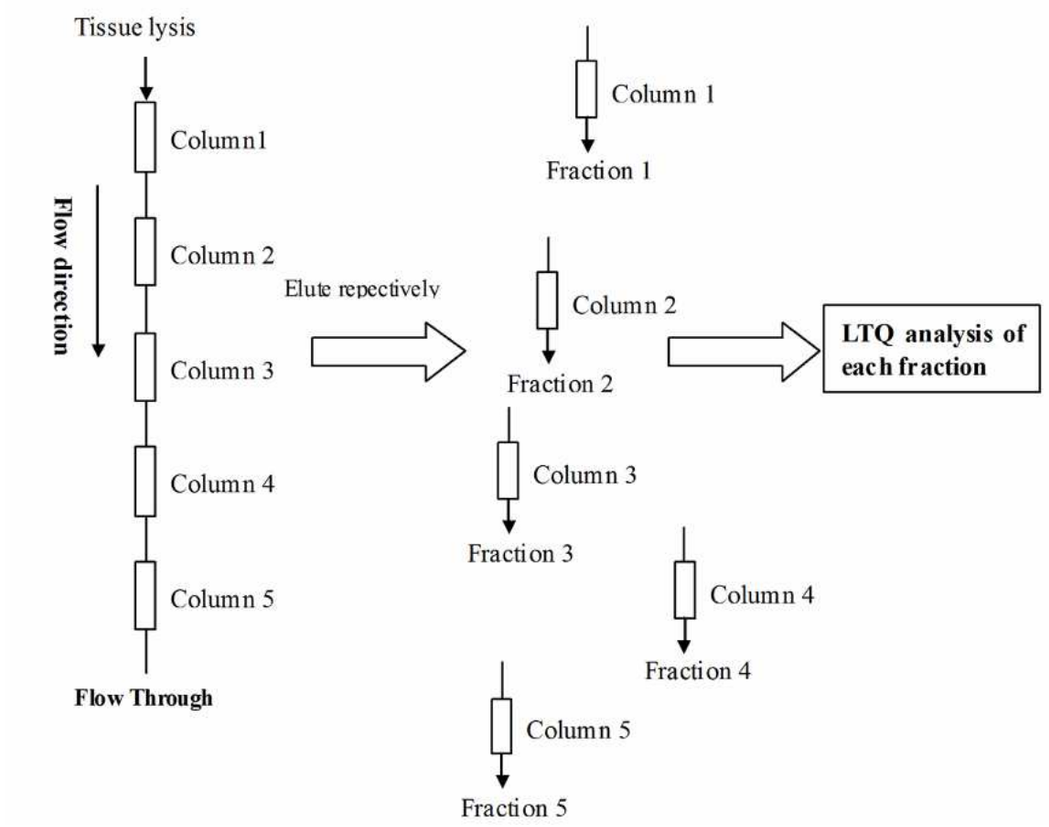


Fig. 1. Schematic illustration of tandem biomimetic affinity fraction used in this work

absorbance characterization to other proteins. At the same time, these ligands showed different absorbance ability. Some ligands exhibited relatively low absorbance ability such as A1-4 and A7-56, but other ligands showed relatively high absorbance ability such as A6, A17-56 and A29-32. According to the affinnity specificity of these ligands, composition of different ligands could be applied in well-distribution of complex tissue proteins. After reducing the complicity of tissue protein samples, more protein information was able to be obtained from proteome analysis. In present research, tandem combination of a few ligands was used as prefractionation of complex protein samples prior to LTQ-MS /MS proteome analysis. A7-56, A84, A11-70, A6, and A29-32 were selected for tandem composition in the follow experiments.

3.3 Tandem biomimetic affinity fractionation and LTQ-MS/MS analysis

The flowchart in FIG. 1 gives a more detailed overview of the tandem procedure, and the ligand column showed relatively low absorbance ability was put front. A7-56, A84, A11-70, A6, and A29-32 were orderly connected. 4mg Cytosol fraction of rat liver proteins was loaded into the tandem columns, after binding and flushing fully 5 tandem columns were divided into single column, and bound protein of each column was respectively eluted by 2-fold column volume of 20mM Glycine-NaOH buffer (pH12.0). Flowthrough (fraction 6) and all the elution fractions (fraction1 to 5) collected were used for subsequent SDS–PAGE electrophoresis (FIG. 2). From proteins band distribution, complex rat liver cytosol proteins were well-distributed into six fractions.

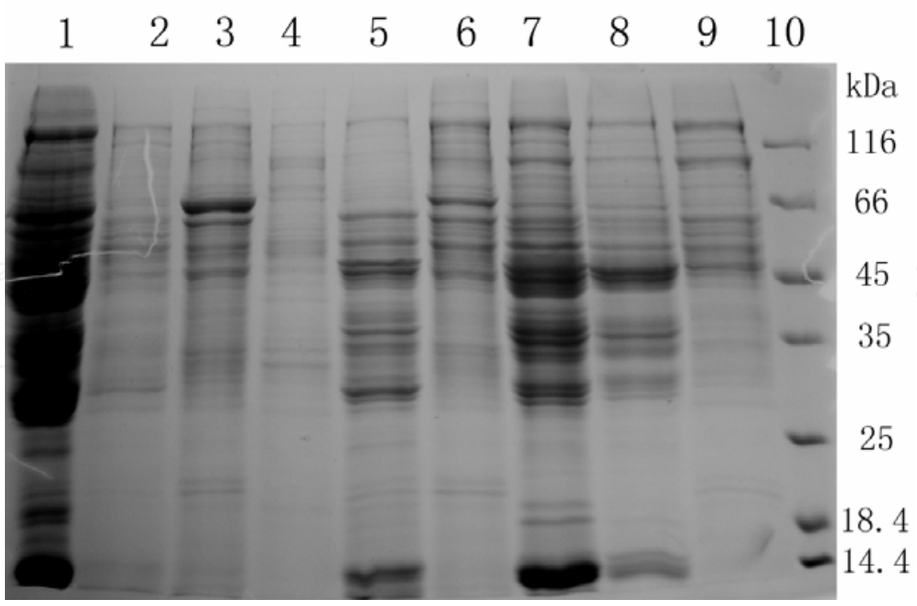


Fig. 2. Partial examples of rat liver proteins absorbance profiles having large band distribution difference. Lane 1 is crude rat liver cytosol. Lane 10 is the protein marker (Fermentas, USA). Lanes2–9show the 20 mM Glycine-NaOH buffer (pH12.0) eluates of columns A1-4, A6, A7-56, A84, A11-70, A17-56, A29-32, and A25-35 respectively.

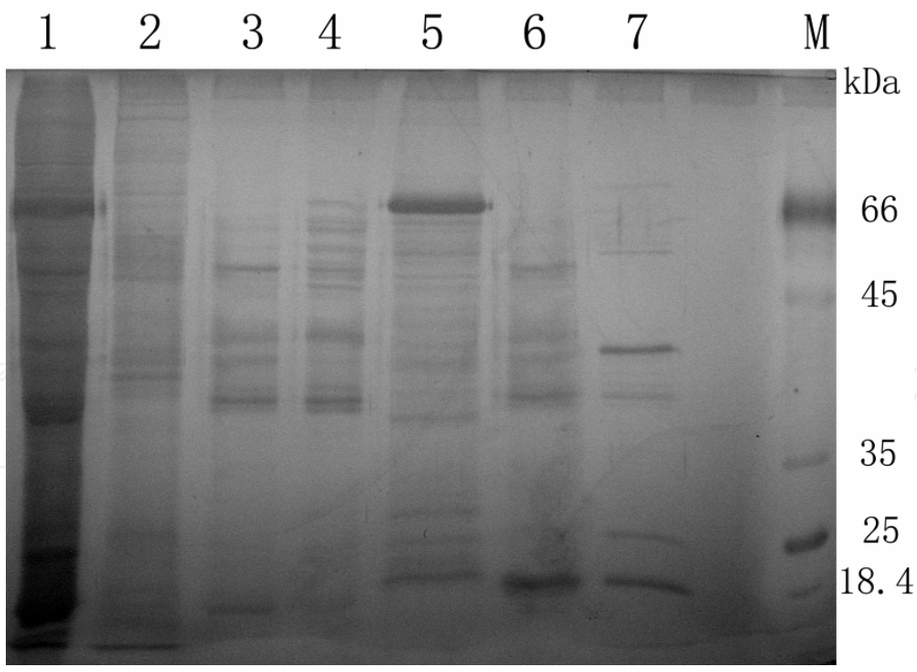


Fig. 3. Protein profiles of tandem affinity fraction. Lane 1 is crude rat liver cytosol. M is the protein marker (Fermentas, USA). Lanes2–6 show the 20 mM Glycine-NaOH buffer (pH12.0) eluates of 5 tandem columns A7-56, A84, A11-70, A6, and A29-32 respectively (fraction 1 to 5). Lane 7 was flowthrough of tandem affinity chromatography (fraction 6).

After being desalted and concentrated using Microcon ultrafiltration membranes (3,000 Nominal Molecular Weight Limit, Millipore), all the fractions collected were used for subsequent LTQ-MS/MS analysis. The technical route was evaluated by comparing the protein numbers detected from the unfractionated cytosol sample with those from six well-distributed fractions. The MS/MS spectra acquired from equivalent normalized aliquots of the six respective fractions were searched against the rat database extracted from SwissProt, GenPept, and PIR entries and a six-way translation of dbEST using the program SEQUEST running on a DECA workstation. In order to avoid false-positive hits, we referred to the parameters reported in previous studies and applied more strict criteria for peptide identification (Charge +1,Xcorr≥1.9; Charge +2, Xcorr≥2.2; Charge +3, Xcorr≥3.75; and DelCN≥0.1). This resulted in highly confident identification of a total of 665 unique rat proteins in the six fractions (Table 1), over eight times as protein numbers detected in unfractionated cytosol sample (only 74 unique proteins). Of these, 290 proteins (827 unique peptides) were characterized in fraction 1(A7-56), 123 proteins (476 unique peptides) in fraction 2(A84), 152 proteins (605 unique peptides) in fraction 3(A11-70), 279 proteins (818 unique peptides) in fraction 4 (A6), 155 proteins (439 unique peptides) in fraction 5 (A29-32), and 60 proteins (344 unique peptides) in fraction 6 (Flow through). At the same time, only 5 proteins were characterized in all the six fractions and 177 proteins were only characterized in fraction1, 24 only in fraction 2, 30 only in fraction 3, 140 only in fraction 4, 42 only in fraction 5, and 17 only in the Flow through fraction (FIG. 4A). Each ligand showed different absorbance characterization to tissue proteins. About 430 unique proteins (64.7%) only characterized in specific fractions, which exhibited well-distribution effect of tandem biomimetic affinity prefractionation. Table 2 showed the number of proteins identified according to 1, 2, 3, and >3 unique peptides. About 40% proteins in each fraction were identified from a unique peptide. It is noted that most of the proteins identified by a single peptide was also only found in a certain fraction, which indicates those proteins are low-abundant components in the cell and enriched after fractionation. FIG. 4B presented sensitivity of this tandem affinity method to a wide variety of protein classes. Of these 665proteins, 61 proteins detected were with extreme size (MW<10kD, or >100kD), 14 with extreme pI value (pI<4.3, or >10), 55 with hydrophobic characterization (GRAVY>0), and 41 with TM domain (Predicted TM Helices≥1). Compared with the traditional 2D-PAGE method, the shotgun strategy presents a number of data with very rapid speed and limited sample consumption. In present research, the tandem affinity prefractionation could obviously enhance the detection ability of shotgun strategy. Most importantly, the tandem affinity prefractionation could be finished in 4 hours, and one-dimension LTQ-MS/MS analysis employed would consumed only 5 hours, which was far lower than 2D-LC-MS/MS. Including other processes (such as concentration, trypsin digestion), only data from single run of 20 h were used in this work.

Fraction	F1(A7-56)	F2(A84)	F3(A11-70)	F4(A6)	F5(A29-32)	F6(FT)	Total
Unique peptides	827	476	605	818	439	344	2413
Unique Proteins	290	123	152	279	155	60	665

Table 1. Peptides and proteins identified in six rat liver tandem fractions using LTQ -MS/MS

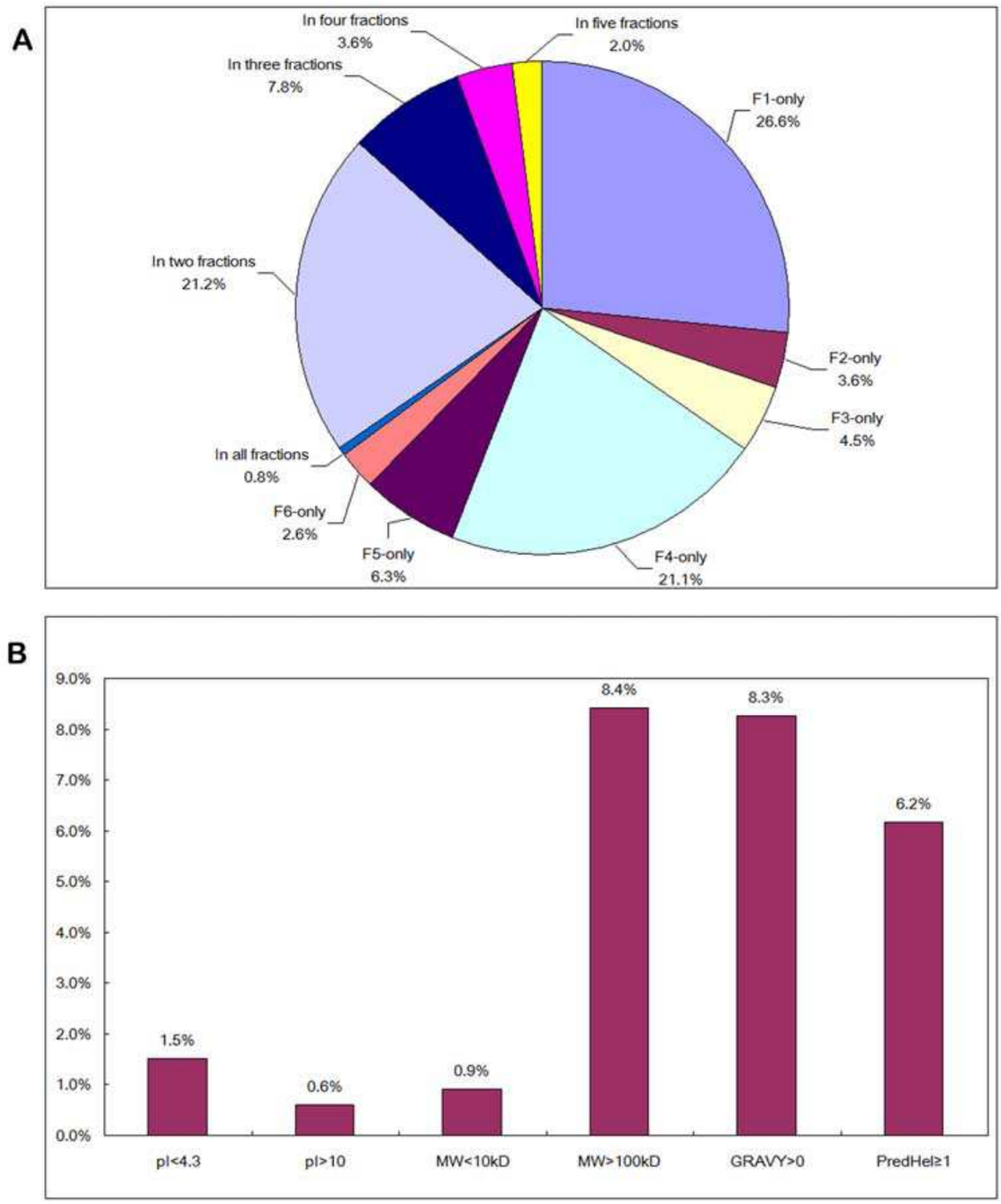


Fig. 4. A total of 665 rat proteins identified from the six tandem fractions of rat liver cytosol. A shows the percentage of the proteins identified from the each fraction and overlapping of different fractions. For example, F1-only represents the proteins identified appeared only in the F1 fraction, not in other fractions; In two fractions means the identified proteins appeared in two different fractions; In three fractions means the identified proteins appeared in three fractions; In all fractions means the identified proteins appeared in all the six fractions. B presents sensitivity to a wide variety of protein classes. The bars indicate the percentage of proteins identified in this study from a variety of protein classes.

Unique Peptides	Total[b]	Fraction[a]											
		F1[c]	F2[c]	F3[c]	F4[c]	F5[c]	F6[c]	F1-only[d]	F2-only[d]	F3-only[d]	F4-only[d]	F5-only[d]	F6-only[d]
1	255	124	39	49	123	62	13	86	13	17	77	22	5
2	122	66	27	23	57	39	9	37	7	7	26	12	5
3	74	31	10	26	24	23	9	16	2	3	9	4	1
>3	214	69	47	54	75	31	29	38	2	3	28	5	6
Total	665	290	123	152	279	155	60	177	24	30	140	43	17

a Six fractions--------F1(A7-56), F2(A84), F3(A11-70), F4(A6), F5(A29-32), F6(Flow Through).
b A total of 665 proteins identified in the six fractions. The peptides attributed to the identification of certain protein in different fractions were combined to calculate the unique peptides.
c Proteins identified in certain fraction.
d Proteins identified only in each fraction.

Table 2. Proteins identified in six tandem rat liver fractions according to different number of unique peptides

3.4 Physicochemical characteristics of the identified proteins

The 665 identified proteins were classified according to different physicochemical characteristics such as molecular mass, pI, hydrophobicity (GRAVY value), and TM domain predicted by TMHMM (FIG. 5).

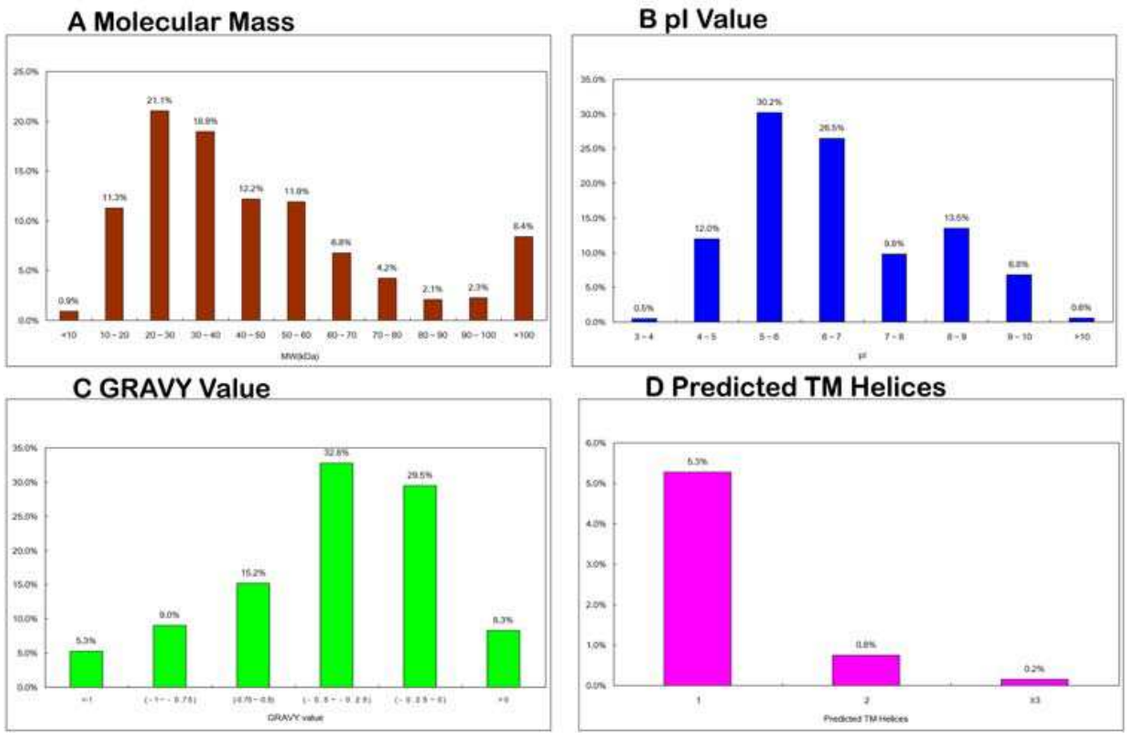


Fig. 5. Distribution of the total proteins identified in relation to their theoretical molecular mass (A), pI (B), GRAVY values (C), and the number of predicted TM helices (D). The bars indicate the percentage of proteins in total proteins identified.

The smallest and the largest molecular mass obtained are 6.6 kDa and 550.8kDa, respectively. For the 665 proteins, 501 proteins (75.3%) distribute among10-60kDa molecular mass intervals, which are compatible with general 2D-PAGE, while there are six(0.9%) proteins with mass <10 kDa and 56 (8.4%) proteins with mass >100 kDa, beyond the general 2D-PAGE separation limits (FIG. 5A). It was more interesting that all proteins with mass <10 kDa only found in a certain fraction, and forty-five (80.4%) proteins with mass >100 kDa only found in a certain fraction, which indicated those proteins also were low-abundant components and enriched after tandem affinity prefractionation.

Regarding the pI distribution, the total 665 proteins distribute across a wide pI range (3.75-11.86) (FIG. 5B). A total of 377 proteins (58%) distribute among pI 5-7 intervals, but only ten (1.5%) proteins have pI <4.3 and four (0.6%) proteins have pI> 10. Interestingly, Most of proteins with extreme pI value were only found in a certain fraction, especially in Fraction 1.

The GRAVY values (http://www.bioinformatics.org/sms2/protein_gravy.html) were determined according to Kyte and Doolittle (1982). The proteins detected in 2D-PAGE gels are generally hydrophilic, thus with negative GRAVY values (37, 38). For the total 665 proteins identified, their GRAVY values vary in the range of –2.04~0.36(FIG. 5C). Interestingly, Fifty-five (8.3%) hydrophobic proteins were identified with positive GRAVY values.

The TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al.,2001) was used to predict protein TM domains. Without specific methods for enrichment or treatment of membrane proteins, we still identified 41(6.2%) proteins of the total 665 proteins have one or more predicted TM domain (FIG. 5B), of which 35 proteins have one TM domain, 5 have two TM domains , and one protein has seven TM domains (FIG. 5D). The protein with seven TM domains was detected as Glucose-dependent insulinotropic receptor (IPI00382339.3, MW=51.7kDa, pI=6.43, GRAVY value=0.36).

In our study, we first combined the tandem biomimetic affinity prefractionation and LTQ-MS/MS (one dimension). From biomimetic affinity ligands library, we screened out some ligands showing large absorbance differences in band distribution to rat liver cytosol proteins. According to the affinnity specificity of these ligands, tandem composition of different ligands could be applied in well-distribution of complex tissue proteins. After reducing the complicity of tissue protein samples from tandem affinity prefractionation, more protein information was able to be obtained from LTQ-MS/MS analysis. With the strict and widely accepted SEQUEST criterion and only the rat protein database, a total of 665 unique rat proteins were identified in the six fractions, which was far higher than proteins indentified in unfractionated cytosol (74 proteins). Only 170 proteins were identified in rat liver or its cytosol sample through 2D-PAGE (Fountoulakis & Suter, 2002). Although the number of proteins identified from all or each rat liver fraction in our study are far from the theoretical protein number of rat liver cytosol, which may have suffered from a limited protein database for rat compared with those of human and mouse.

In present research, the tandem affinity prefractionation could obviously enhance the detection ability of shotgun strategy. Most importantly, the tandem affinity prefractionation could be finished in 4 hours, and one-dimension LTQ-MS/MS analysis employed would consumed only 5 hours, which was far lower than 2D-LC-MS/MS. Including other processes (such as concentration, trypsin digestion), only data from single run of 20 h were used in this work.

4. Prefractionation of of rat liver cytosol proteins utilizing cascade composition of affinity chromatography

4.1 Cascade affinity prefractionation of tissue samples

From biomimetic affinity ligands library in our lab, we first screened out some affinity ligands showing large absorbance differences in band distribution to rat liver cytosol proteins. Then, the affinity ligands having medium absorbance ability were selected, cascade composition of these affinity ligands could be adopted for the prefractionation of tissue proteins. In this study, cascade composition of three affinity ligands was adopted, and the schematic illustration of the workflow was shown in FIG. 6. Briefly, after complex tissue sample was loaded to the first affinity ligand column, flowthrough and elution were collected for the two new fractions. Then, after above two new fractions were parallel loaded to the second affinity liand column, flowthrough and elution were collected for the four new fractions. At last, above four new fractions were loaded to the third affinity ligands, and 8 new fractions could be obtained. The binding and elution conditions were same as above. After being desalted and concentrated using Microcon ultrafiltration membranes (3,000 Nominal Molecular Weight Limit, Millipore), all the fractions collected were used for subsequent SDS–PAGE electrophoresis and LTQ-MS/MS analysis.

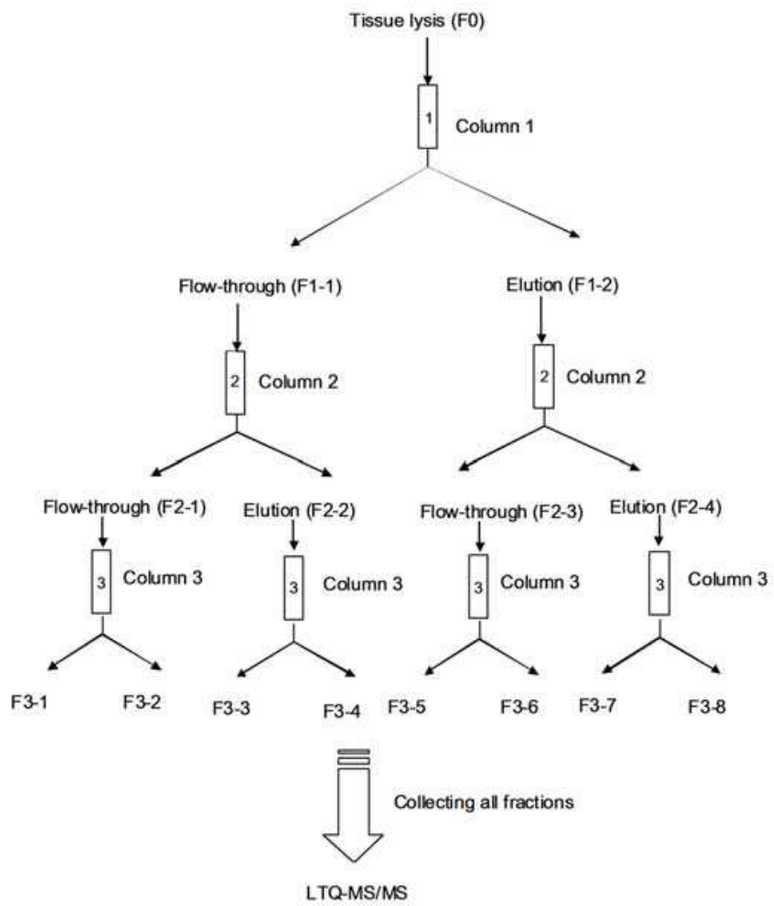


Fig. 6. Schematic illustration of Cascade affinity fraction used in this work

4.2 Evaluating for differential protein absorbance profiles of mimetic affinity ligands

After analyzing the screening data of our lab in serum and leech proteins, we selected the affinity ligands having medium absorbance ability. Sixty affinity ligands were selected from the affinity ligands library of our lab, and the protein absorbance characterization of these ligands was reevaluated using rat liver cytosol sample as materials. From the SDS-PAGE profiles of each ligand, we found that some ligands showed large absorbance differences in band distribution to rat liver cytosol proteins (FIG.7). Partial examples of proteins absorbance profiles were shown in FIG.7, and each ligand showed its own specific binding ability to some group of proteins, but low absorbance characterization to others. At the same time, some affinity ligands exhibited relatively low absorbance ability such as A11-70 and A7-56, but other ligands showed relatively high absorbance ability such as A6, A15 and A29-32. According to the affinnity specificity of these ligands, composition of different ligands could be used to fractionate complex tissue proteins into different group of proteins. After reducing the complicity of tissue protein samples, more protein information was able to be obtained from proteome analysis. In present research, cascade combination of several affinity ligands was used as prefractionation of complex protein samples prior to LTQ-MS /MS proteome analysis. Three affinity ligands (A15, A8-54, A11-70) were selected for three-cascade composition in the follow experiments.

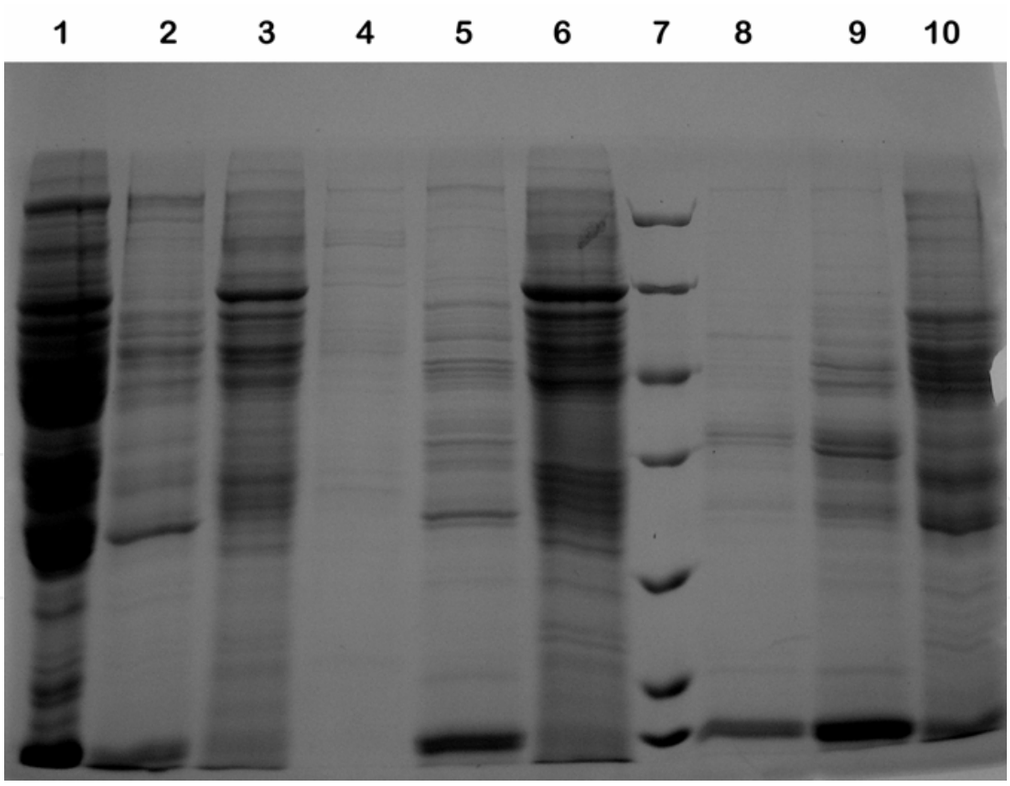


Fig. 7. Partial examples of rat liver proteins absorbance profiles having large band distribution difference. Lane 1 is crude rat liver cytosol. Lane 7 is the protein marker: 116, 66.2, 45, 31, 25, 18.4, 14.4kDa (Fermentas, USA). Lanes2–6, 8-10show the 20 mM Glycine-NaOH buffer (pH12.0) eluates of columns A1-4, A6, A7-56, A8-54, A15, A11-70, A22-83, and A25-35, respectively.

4.3 Cascade affinity fractionation and LTQ-MS/MS analysis

The flowchart in FIG. 6 gave a more detailed overview of the cascade fractionation procedure. Affinity ligand column A15, having relatively high absorbance ability, was put in the first affinity fractionation. After 4mg rat liver cytosol was loaded to the column A15, the flow-through and elution were collected (adjusted to pH 7.0 instantly), named as F1-1 and F1-2. Then, F1-1 and F1-2 were parallel loaded to the column A8-54, four fractions (F2-1~F2-4) were obtained from collecting the flow-through and elution. At last, the four fractions in the second affinity fractionation were loaded to A11-70, eight new fractions (F3-1~F3-8) were obtained from collecting the flow-through and elution. All fractions were collected for subsequent SDS–PAGE electrophoresis (FIG. 8). From proteins band distribution profiles in FIG. 8, the three-cascade-fractionation reduced the complexity of tissue sample gradually. In the end, complex rat liver cytosol proteins were well-distributed into eight simple fractions (F3-1~F3-8), and each fraction exhibited its own specific proteins distribution characterization.

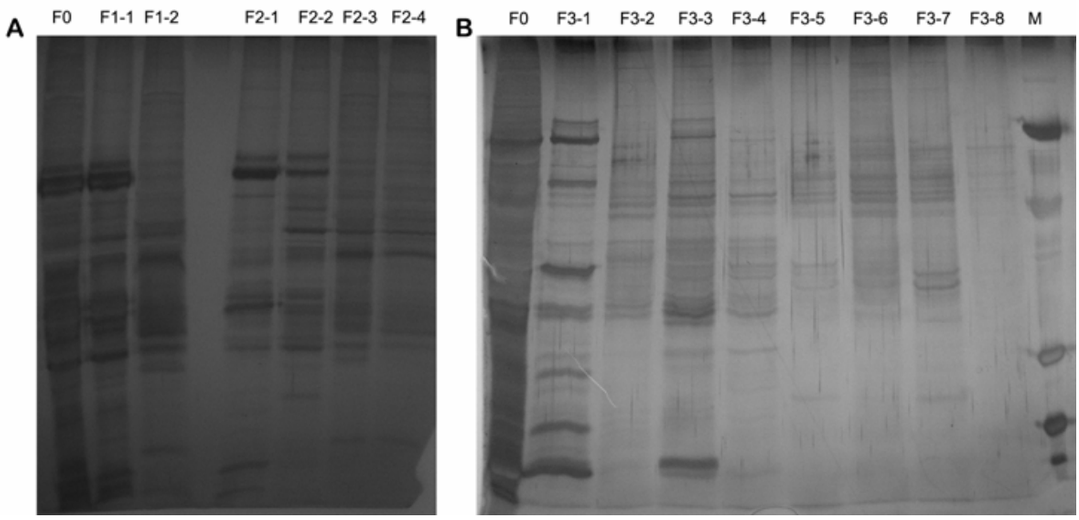


Fig. 8. Protein profiles of cascade affinity fractionation (A15~A8-54~A11-70). A shows the proteins distribution of all the fractions in the first and second fractionation, F0 is crude rat liver cytosol, F1-1 and F1-2 are flowthrough and elution of A15, F2-1~F2-4 are four new fractions of the second fractionation(A8-54). B shows the proteins distribution of all the fractions in the third fractionation, F0 is crude rat liver cytosol, F3-1~F3-8 are eight new fractions of the third fractionation (A11-70), M is the protein marker: 116, 66.2, 45, 31, 25, 18.4, 14.4kDa (Fermentas, USA).

After being desalted and concentrated using Microcon ultrafiltration membranes (3,000 Nominal Molecular Weight Limit, Millipore), all the fractions collected were used for subsequent LTQ-MS/MS analysis. The technical route was evaluated by comparing the protein numbers detected from the unfractionated rat liver cytosol sample with those from the fractions of each grade in the cascade affinity fractionation. The MS/MS spectra acquired from equivalent normalized aliquots of the 15 respective fractions were searched against the IPI rat database using the program SEQUEST running on ISB/SPC Proteomics workstation. As most sequence search engines return results even for 'unmatchable' spectra, proteome researchers must devise ways to distinguish correct from incorrect peptide

identifications. The target-decoy search strategy represents a straightforward and effective way to manage this effort. In this work, The ISB/SPC Proteomics Tools-TPP V4.2 was applied, which combined the decoy database searching approach with automated filter criteria optimization. Peptides with a p value of equal or bigger than 0.95, protein probability with a p value of equal or bigger than 0.99, were used for filtering conditions. Three hundred ninety-one unique proteins were identified in unfractionated rat liver cytosol, 499 unique proteins were identified (27.6% increased) in two fractions (F1-1and F1-2) of the first affinity fractionation, 616 unique proteins were identified (23.4% increased) in four fractions (F2-1~F2-4) of the second affinity fractionation, and 732 unique proteins (18.8% increased) were identified in eight fractions (F3-1~F3-8) of the third affinity fractionation (Table 1). In the end, the cascade affinity fractionation resulted in highly confident identification of a total of 859 unique rat protein groups in cascade affinity fractions (Table 1), over two times as protein numbers detected in unfractionated rat liver cytosol sample. It was noted that most of the proteins identified in unfractionated sample (364 proteins, 93.1%) also emerged in the cascade affinity fractions, and 495 new proteins were identified in the cascade affinity fractions. Table 2 showed the increasing percentage of protein numbers identified in all affinity fractionation processes, and the effect of each fractionation was obvious. The minimal increasing percentage was 14.4%, and the maximum of increasing percentage was 113.3%. Therefore, the cascade affinity fractionation resulted in the highly increasing of the protein numbers identified, and much more new proteins were detected.

Fraction	F0[a]	F1-1~F1-2[b]	F2-1~F2-4[c]	F3-1~F3-8[d]	Total[e]
Unique peptides	1511	1432	1637	1686	1951
Unique Proteins	391	499	616	738	859

a F0 was unfractionated rat liver cytosol.
b Two fractions(F1-1~F1-2) in the first affinity fractionation.
c Four fractions(F2-1~F2-4) in the second affinity fractionation.
d Eight fractions(F3-1~F3-8) in the third affinity fractionation.
e Total proteins identified in all fractions(no including F0).

Table 3. Peptides and proteins identified in all rat liver fractions of the cascade affinity fractionation using LTQ-MS/MS

First[a]	F0	F1-1	F1-2	F2-1	F2-2	F2-3	F2-4
	391	298	374	360	165	302	359
Second[b]	F1-1+F1-2	F2-1+F2-2	F2-3+F2-4	F3-1+F3-2	F3-3+F3-4	F3-5+F3-6	F3-7+F3-8
	499	387	443	412	352	353	430
Increasing (%)	27.6	29.9	18.4	14.4	113.3	16.9	19.8

a First showed the protein numbers identified in the fractions before affinity fractionation .
b Second showed the protein numbers identified in the collected fractions (including flow-through and elution) after affinity fractionation.

Table 4. Increasing percentage of protein numbers identified in all affinity fractionation processes

Table 5 showed the protein numbers identified according to 1, 2, 3, and >3 unique peptides. About 40% proteins in each fraction were identified from a unique peptide, and a total of 497 unique protein groups (56.09%) in all fractions were identified from a unique peptide. About 20% proteins in each fraction were identified from two unique peptides, 10% from three unique peptides, and 15~30% from >3 unique peptides. It was noted that most of the proteins identified from two or more unique peptides were often repeated in different fractions, which indicated those proteins are high-abundant components in the cell. On the contrary, the proteins identified by a single peptide showed relatively low repeated proportion, which indicated that most of those proteins were low-abundant components in the cell and enriched after fractionation. Comparing with the traditional 2D-PAGE method, the shotgun strategy presents a number of data with very rapid speed and limited sample consumption. In present research, the cascade affinity prefractionation could obviously enhance the detection ability of shotgun strategy. Most importantly, the tandem affinity prefractionation could be finished in 8 hours, and one-dimension LTQ-MS/MS analysis employed would consumed only 5 hours, which was far lower than 2D-LC-MS/MS. Including other processes (such as concentration, trypsin digestion), only data from single run of 24 h were used in this work.

Unique Peptides	Total[b]	Fraction [a]														
		F0	F1-1	F1-2	F2-1	F2-2	F2-3	F2-4	F3-1	F3-2	F3-3	F3-4	F3-5	F3-6	F3-7	F3-8
1	479	122	120	141	159	78	136	128	126	119	105	87	152	93	101	147
2	154	79	59	82	58	37	53	57	55	56	55	49	53	48	57	76
3	85	43	32	48	42	16	41	53	20	35	28	26	31	31	32	45
>3	141	147	87	103	101	34	72	121	78	68	81	78	52	56	62	114
Total	859	391	298	374	360	165	302	359	279	278	269	240	288	228	252	382

a fifteen fractions - F0 was unfractionated rat liver cytosol. F1-1 and F1-2 were two fractions in the first affinity fractionation. F2-1~F2-4 were four fractions in the second affinity fractionation. F3-1~F3-8 were eight fractions in the third affinity fractionation.

b A total of 859 proteins identified in the fourteen fractions(no including F0). The peptides attributed to the identification of certain protein in different fractions were combined to calculate the unique peptides.

Table 5. Proteins identified in all fractions of cascade affinity fractionation of rat liver cytosol according to different number of unique peptides

4.4 Physicochemical characteristics of the identified proteins

The 859 identified proteins were classified according to different physicochemical characteristics such as molecular mass, pI, hydrophobicity (GRAVY value), and TM domain predicted by TMHMM (FIG. 9).

The smallest and largest molecular mass obtained are 6.1kDa and 419.6kDa, respectively. For the 859 proteins, 617 proteins (71.83%) distribute among 20-70kDa molecular mass intervals, which are compatible with general 2D-PAGE, while there are 75 proteins (8.73%) with mass <20kDa and 73 proteins (8.5%) with mass >100kDa, beyond the general 2D-PAGE separation limits (FIG. 9A). Regarding the pI distribution, the total 859 proteins distribute across a wide pI range (3.75-11.56) (FIG. 9B). A total of 594 proteins (69.16%) distribute among pI 5-8 intervals, but only 85 proteins (9.9%) have pI <5 and 180 proteins (20.95%) have pI> 8.

The GRAVY values (http://www.bioinformatics.org/sms2/protein_gravy.html) were determined according to Kyte and Doolittle (Kyte and Doolittle, 1982). The proteins detected in 2D-PAGE gels are generally hydrophilic, thus with negative GRAVY values (Fountoulakis, and Takacs, 2001; Fountoulakis and Suter, 2002). For the total 859 proteins identified, their GRAVY values vary in the range of –2.047~0.322(FIG. 9C). It is noted that most of the proteins are hydrophilic (787 proteins with negative GRAVY values), and 72 (8.38%) hydrophobic proteins were identified with positive GRAVY values.

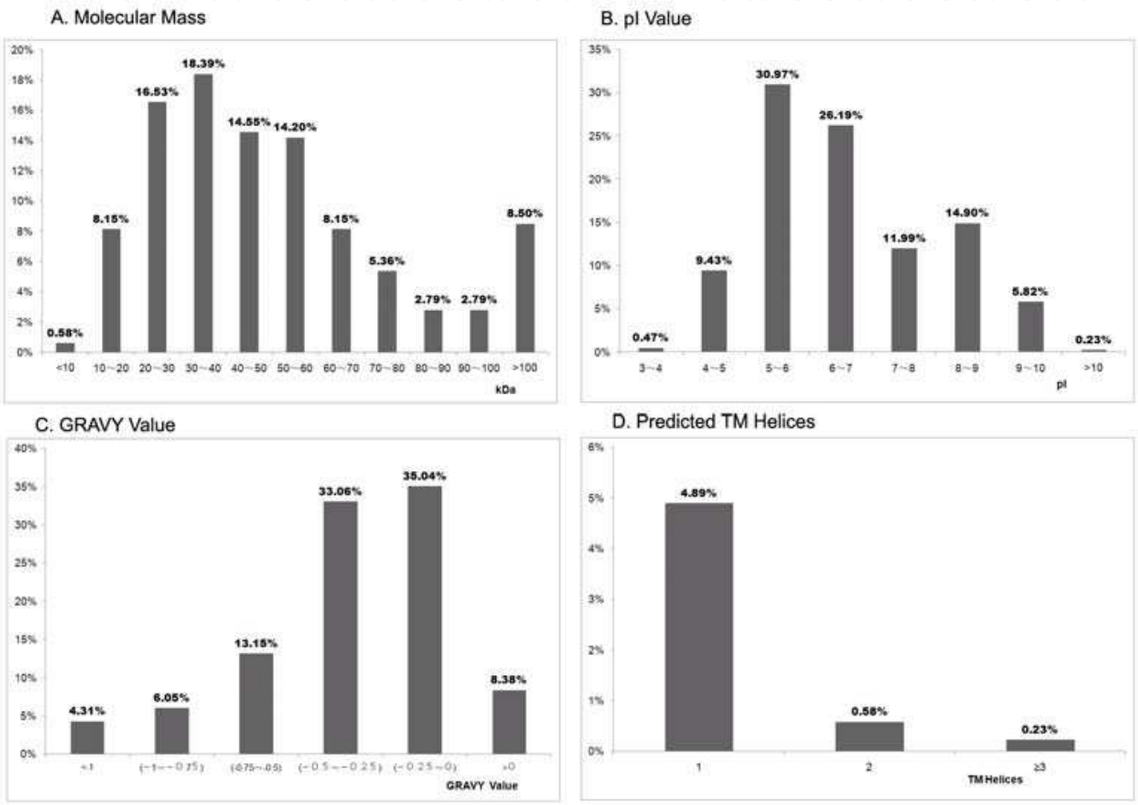


Fig. 9. Distribution of the total proteins identified in relation to their theoretical molecular mass (A), pI (B), GRAVY values (C), and the number of predicted TM helices (D). The bars indicate the percentage of proteins in total proteins identified.

Analysis of membrane proteins is an important field in proteomics because membrane proteins are represented by 30% of the genome and constitute approximately 70% of all human protein based drug targets (Wallin and von Heijne, 1998; Hopkins and Groom, 2002). Analysis of membrane proteins has been notoriously difficult, which has been demonstrated by their under-representation in 2D gels (Molloy et al., 1998; Santoni et al., 2000). The TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict protein TM domains of membrane proteins (Krogh et al., 2001). Without specific methods for enrichment or treatment of membrane proteins, we still identified 49(5.7%) proteins of the total 859 proteins have one or more predicted TM domain (FIG. 9D). Of these, 42 proteins have one TM domain, 5 have two TM domains, and two proteins have three TM domains (FIG. 9D).

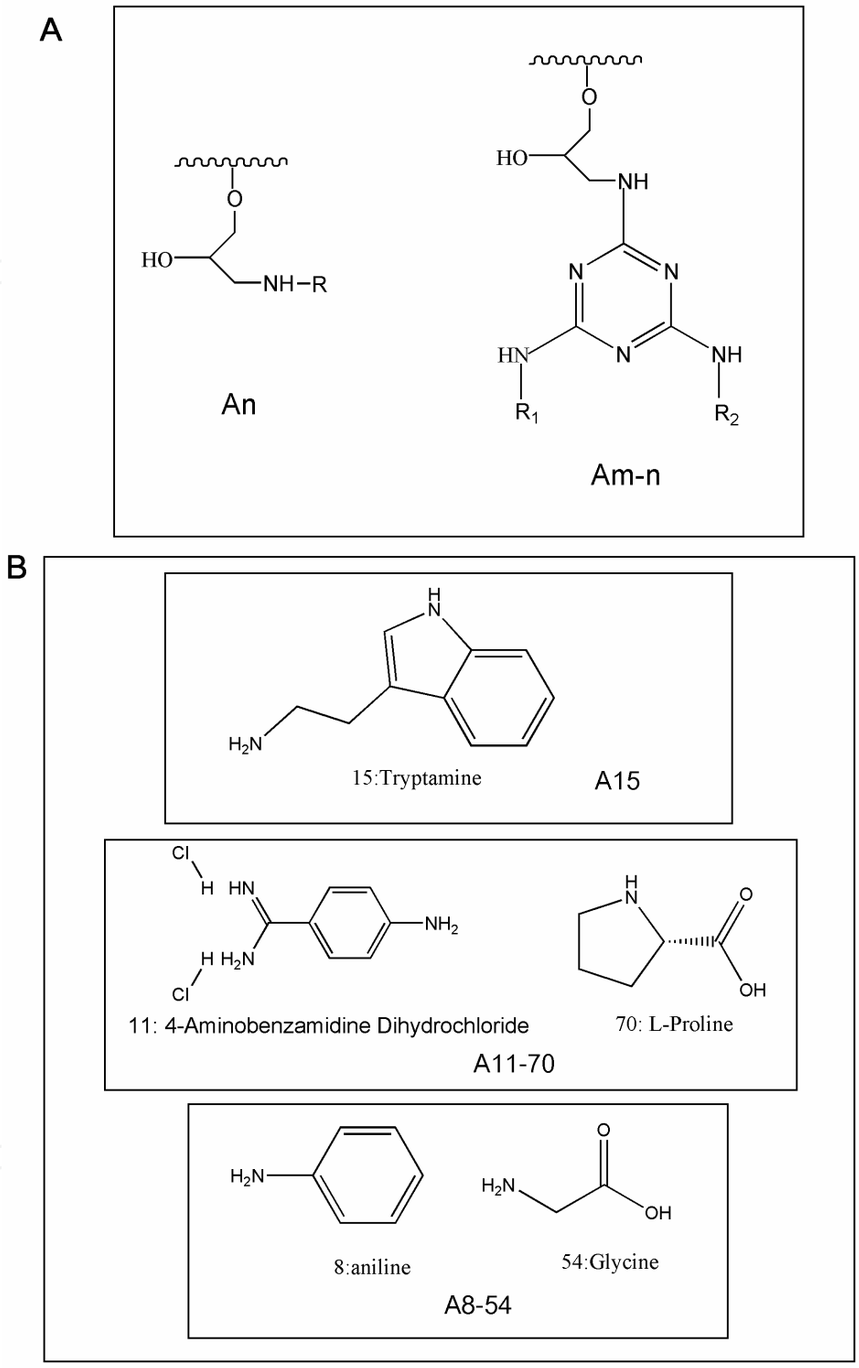


Fig. 10. The structure of affinity ligands used in cascade affinity prefractionation. Sepharose 4B was matrix, which was activated with epichlorohydrin. A showed two kinds of synthetic ligands: first, single amino compound was directely linked to activated sepharose, named as An; second, cyanuric chloride was coupled to activated sepharose as a large spacer, and two chloride in spacer could be substituted by two amino compounds, named as Am-n. B gave the chemical structures of amino compounds used in three selected affinity ligands (A15, A11-70, A8-54).

5. Discussion

In our study, we first combined the cascade biomimetic affinity prefractionation with LTQ-MS/MS analysis. From biomimetic affinity ligands library, we screened out three ligands showing large absorbance differences in band distribution to rat liver cytosol proteins. The structures of the these elected ligands were shown in Fig. 10, and the structure difference was obvious. According to the difference of these ligands in size, shape, structure, and biochemical characterization, each ligand could exhibit the affinity specificity to some protein groups. Therefore, the cascade composition of different ligands could be applied in well-distribution of complex tissue proteins. After reducing the complicity of tissue protein samples, more protein information was able to be obtained from LTQ-MS/MS analysis. In this report, only three ligands were used in the cascade composition of affinity chromatography. However, after simple affinity fractionation for three times, the crude rat liver cytosol was fractionated into eight relatively simple fractions, and a total of 859 unique rat protein groups were identified in the cascade affinity fractions, which was far higher than proteins indentified in unfractionated cytosol (391 proteins). According to previous research reports, in rat liver cytosol sample Only 170 proteins were identified through 2D-PAGE (Fountoulakis and Suter, 2002), and 222 proteins were identified through 2D-LC-MS/MS (Jiang et al., 2004). We applied much more strict filtering condition compared with previous references, but our results gave more non-redundant rat liver cytosol protein groups (859).

In present research, the cascade affinity fractionation could obviously enhance the detection ability of shotgun strategy. For more complex tissue samples, much more affinity ligands could be selected fot cascade composition. Combined usage of the cascade affinity fractionation and LTQ-MS/MS was simple, low-cost, and effective, which gave a broad application prospect in proteomics.

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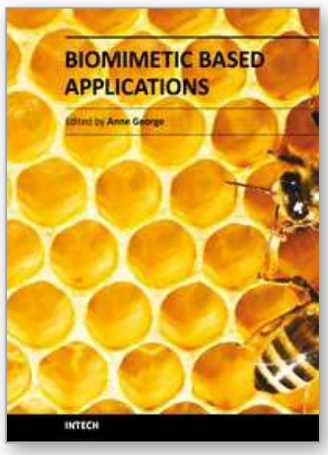
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