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Characterization of Molecules Binding to the 70K N-terminal Region of Fibronectin by IFAST Purification Coupled with Mass Spectrometry

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Abstract

Fibronectin (Fn) is a large glycoprotein present in plasma and extracellular matrix and is important for many processes. Within Fn the 70kDa N-terminal region (70k-Fn) is involved in cell-mediated Fn assembly, a process that contributes to embryogenesis, development, and platelet thrombus formation. In addition, major human pathogens including *Staphlycoccus aureus* and *Streptococcus pyogenes*, bind the 70k-Fn region by a novel form of protein-protein interaction called β-zipper formation, facilitating bacterial spread and colonization. Knowledge of blood plasma and platelet proteins that interact with 70k-Fn by β-zipper formation is incomplete. In the current study, we aimed to characterize these proteins through affinity purification. For this affinity purification, we used a novel purification technique termed immiscible filtration assisted by surface tension (IFAST). The foundation of this technology is immiscible phase filtration, using a magnet to draw paramagnetic particle (PMP)-bound analyte through an immiscible barrier (oil or organic solvent) that separates an aqueous sample from an aqueous eluting buffer. The immiscible barrier functions to remove unbound proteins via exclusion rather than dilutive washing used in traditional isolation methods. We identified 31 interactors from plasma, of which only seven were previously known to interact with Fn. Furthermore, five proteins were identified to interact with 70k-Fn from platelet lysate, of which one was previously known. These results demonstrate that IFAST offers advantages for proteomic studies of interacting molecules in that the technique requires small sample volumes, can be done with high enough throughput to sample multiple interaction conditions, and is amenable to exploratory mass spectrometric and confirmatory immuno-blotting read-outs.

Keywords

Fibronectin; IFAST; Interactome; Myosin Heavy Chain-9; Gelsolin

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INTRODUCTION

Protein-protein interactions regulate various cellular processes from DNA replication and transcription to mRNA translation, intracellular signaling, and interactions with extracellular cues. Cataloguing these interactions is a major challenge. Various techniques have been used to study protein-protein interactions.[1-12] A mass spectrometric read-out after affinity purification (AP-MS) is a favored technique for analysis of the proteins that interact with a given target, the interactome for that target, in physiologically relevant conditions.[13-20]

A crucial aspect of AP-MS is availability of a purification technique that is simple, efficient, well controlled, and amenable to high-throughput approaches. The initial step in this process is tight linkage of the target protein (i.e. bait protein) to a solid phase. A number of linkage strategies have been developed.[8, 21, 22] The biotin-avidin system offers sub-nM affinity that is stable in a variety of solvent conditions.[12, 23] In this approach, the bait protein is tagged with biotin and bound to an avidin-coated solid phase so that interacting and non-interacting proteins in a mix of possible interacting proteins can be separated by washing of the solid phase.[24, 25] It is critical that the biotin tagging be accomplished in a manner that does not affect possible interactions of the bait protein with the interacting proteins.

Currently, most affinity purification techniques require multiple wash steps to remove non-interacting proteins.[13, 26] Important protein-protein interactions may be transient and potentially missed with purifications that involve multiple washes. To overcome this limitation, we have developed a new strategy of affinity purification for detection of physiologically relevant interactions, immiscible filtration assisted by surface tension (IFAST).[27-31] The foundation of this technology is immiscible phase filtration, using a magnet to draw paramagnetic particle (PMP)-bound analyte through an immiscible barrier (oil or organic solvent) that separates an aqueous sample from an aqueous eluting buffer.[32, 33] The boundary between the aqueous and oil phase forms a virtual wall, which requires energy to be overcome. Therefore, only molecules interacting with the PMPs are able to cross. The IFAST device used in the present study has three different aqueous wells, input, wash, and elution. The aqueous wells are separated by oil barriers that separate PMP-bound proteins from unbound proteins.

Fibronectin (Fn) is a large glycoprotein present in plasma and extracellular matrix (ECM) and is important for many pathophysiologic processes.[34] Fn contains binding domains for fibrin, collagen and heparan sulfate, and a RGD sequence that interacts with integrins.[35] Within the structure of Fn, the 70kDa N-terminus (70k-Fn) region is involved in cell-mediated Fn assembly, a process required for embryogenesis, development, and platelet thrombus formation.[36, 37] In addition, a large number of bacterial surface proteins interact with 70k-Fn by an unusual type of protein-protein interaction called β-zipper formation.[38] Bacteria using this interaction include major human pathogens including *Staphylococcus aureus, Streptococcus pyogenes*, and *Borrelia burgdorferi*.[36, 38-41] Currently, type I collagen is the only human protein known to interact with 70k-Fn by β-zipper formation.[42, 43] In this paper we report the first use of IFAST for AP-MS studies and focus on defining the interacting partners of 70k-Fn from plasma and platelets. IFAST performed as well or better than multi-wash purification in detecting proteins known to interact with 70k-Fn. Furthermore, IFAST coupled with nHPLC-MS/MS detected previously unknown interactions of proteins from plasma or platelets with 70k-Fn.

MATERIALS AND METHODS

Recombinant 70k-Fn and 70k-Fnbap

70k-Fn, which encompasses the N-terminus through the 9th type I module of Fn (residues 32-608 numbering from the initiating methionine), was expressed in insect cells as previously described.[37] 70k-Fnbap (biotin acceptor peptide) is 70k-Fn with addition at its C-terminus of a 21 amino acid sequence LERAPGGLNDIFEAQKIEWHE that is recognized by BirA biotin protein ligase,[44] thus allowing site-specific enzymatic mono-biotinylation of the lysine within that sequence. A DNA segment encoding the sequence was cloned into 70k-Fn directly after the His6 tag, and the resulting 70k-Fnbap protein was expressed in insect cells in the same conditions as 70k-Fn.

Functional Upstream Domain (FUD) and d32-36

The 49-residue fibronectin-binding polypeptide known as functional upstream domain (FUD) and inspired by the F1 adhesin of *Strepcoccus pyogenes* and the deletion mutant of FUD lacking the 32nd-36th amino acids (d32-36) were produced in bacteria as described.[36] FUD was labeled with FITC as described.[45]

Chemical biotinylation

EZ-Link N-hydroxysulfosuccinimide-biotin (Pierce/Thermo Scientific, Rockford, IL) was used to chemically biotinylate 70k-Fn (ChemB70k) and bovine serum albumin (ChemBBSA) using the manufacturer's suggested protocol. Biotinylation of ChemB70k was verified by adding the protein to microtiter wells coated with 10 μg/ml gelatin and probing with streptavidin conjugated to alkaline phosphatase. Monoclonal antibody 4D1 to 70k-Fn[36] was used to quantify the total 70k-Fn present in the wells.

Enzymatic biotinylation of 70k-Fnbap

70k-Fnbap was enzymatically biotinylated *in vitro* using BirA biotin protein ligase kit (Avidity, Aurora, CO) with modifications to the manufacturer's instructions. Purified 70k-Fnbap (0.7 mg) was added to 0.5 ml gelatin-agarose (Sigma Aldrich Corp., St Louis, MO) and incubated for 1 hr at ambient temperature. Unbound protein was washed away from the gelatin-agarose with TBS (10mM Tris, 300mM sodium chloride, pH 7.4) followed by washing and equilibration with 1 M potassium glutamate (L-glutamic acid mono-potassium salt) pH 8.0. One part Biomix-A (Avidity, Aurora, CO), 1 part Biomix-B (from the kit provided by Avidity), 8 parts 1M K-glutamate pH 8.0, and 8 μg of BirA Biotin Protein Ligase was added to the 70k-Fnbap bound to gelatin-agarose and incubated at 30°C for 2 hr with gentle mixing. The gelatin-agarose was then washed with 2 column volumes of 1 M potassium glutamate pH 8.0, followed by 5 column volumes of TBS. Labeled 70k-Fnbap (EnzB70k) was eluted from the gelatin-agarose with 3M guanidine hydrochloride in TBS, and then dialyzed into TBS containing 1M sodium bromide. Biotinylation was confirmed by adding the protein to microtiter wells coated with 10 μg/ml gelatin and probing with streptavidin conjugated to alkaline phosphatase. The monoclonal antibody 4D1 was used to quantify the total 70k-Fn present in the duplicate wells.

Plasma-serum and platelet lysate

Blood was drawn from healthy donors with prior approval from the University of Wisconsin Institutional Review Board. Six volumes of blood was mixed with 1 volume of acid-citrate dextrose (ACD, NIH formula-A) and centrifuged at 250g for 20 min to obtain platelet-rich plasma (PRP). The PRP was centrifuged at 700g for 20 min to sediment the platelets, and the platelet-poor plasma (PPP) was removed leaving the platelets behind. Calcium chloride and thrombin were added to the PPP at final concentrations of 20 mM and 1 U/mL

respectively. This mixture was incubated at 37°C for 2 hr to allow for clot formation. The clot was removed with a sterile Pasteur pipet, and plasma-serum was heated to 56°C for 3 min, sterile filtered, flash frozen and stored at -80°C until use.

Alternatively, prostaglandin E1 (PGE_1) (Sigma Aldrich, St Louis, MO) was added to PRP at a final concentration of 20 ng/mL. After a 15-min incubation, the PRP was centrifuged at 700g for 20 min to sediment the platelets. After 3 washes with Hepes wash buffer ($1/10^{th}$ volume ACD-A, 50mM Hepes (FW 238), 150mM sodium chloride, 5mM dextrose, pH 7.6) containing 20ng/mL PGE_1, the platelet pellet was resuspended in Hepes wash buffer without PGE_1 at a concentration of 1.11×10^9/mL. The platelets were lysed by adding $1/10^{th}$ volume of 10% triton-X100 in Hepes wash buffer resulting in final concentrations of 1% triton-X100 and 1.0×10^9 platelets/mL. Cellular debris was removed by centrifugation at 16,000g for 30 min. The lysate was flash-frozen and stored at -80°C until use.

Fluorescence polarization

Fluorescence polarization measurements were preformed in Tecan GENios Pro multifunctional microplate reader (Tecan Austria GmbH). The reactions were in Tris-buffered saline (TBS) containing 100 mM sodium chloride and 0.1% BSA at 25°C. Excitation and emission wavelengths were 485 and 535 nm, respectively. Unlabeled 70k-Fn, EnzB70k, or ChemB70k was mixed with FITC-FUD in equal volume. Final concentrations were 5, 10, 20, 50, or 100 nM 70k-Fn and 20 nM FITC-FUD. After mixing, each group was incubated for 2 hr before polarization of FITC-FUD was monitored.

IFAST device

Injection compression molded polypropylene IFAST devices were generously donated by Tye Gribb (Madison, WI). IFAST consists of wells containing the aqueous phase separated by oil containing wells, wherein the first aqueous well contains the unpurified protein mix, and the last aqueous well contains the elution buffer. Unless otherwise identified, all experiments used the one-wash-well IFAST. To compare the role of multiple aqueous wells on the detected protein bands, some experiments included the two-wash-well IFAST.

IFAST Protein Purification

PMPs coated with strepavidin [Dynabeads M-280, Life Sciences], 40 μl of resuspended slurry, were washed with PBS and resuspended in TBS containing 150 mM sodium chloride and 0.1% BSA. ChemB70k, EnzB70k, or ChemBBSA, 1.3 μg in 10 μl was added to the PMPs to give a final concentration of 3 μM and left for 30 min with occasional swirling to resuspend the PMPs. Unbound protein was removed by washing PMPs three times with TBS containing 0.1% BSA and resuspended in 40 μl TBS with or without 0.1% BSA. Charged PMPs were then incubated for 30 min with 10 μl of solution containing potential ligands with occasional swirling. In some experiments, 1 μM FUD was also present in the incubation of charged PMPs and potential ligand. Before the mixture was placed in the IFAST input well, 15 μl of 50 mM ammonium bicarbonate was place in the wash well(s), and 10 μl of eluting buffer (1% acetic acid) was placed in the output well. After filling the input well, 12 μl of the fluorinated oil FC40 (3M, Minneapolis, MN) was placed in the intervening oil wells. NdFeB magnets (K&J Magnetics, #B333-N52) were used to draw the PMPs through the immiscible phase barriers. The magnet was moved by hand at the approximate rate of 3 mm/sec.

Manual Wash Experiments

Charged PMPs, 40 μl, were incubated for 30 min with 10 μl of solution containing potential ligands, with occasional swirling, after which the mixture was washed 1-3 times in

microcentrifuge tubes. For each wash, PMPs were collected with a magnet on the side of the tube after ~1 min, supernatant was aspirated, and PMPs were resuspended in 50 mM ammonium bicarbonate. After the last wash, PMPs were suspended in 10 μl 1% acetic acid, and proteins released from the beads were collected.

SDS-PAGE

Samples were analyzed under reducing conditions on 8% acrylamide slab gels and silver-stained, stained with Gelcode blue (Pierce), or Western blotted as previously described. [46, 47] Antibodies for Western blotting included monoclonal mouse anti-gelsolin (BD Biosciences, San Jose, CA) and monoclonal rabbit anti-MyH9 (Abcam, Cambridge, MA).

Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS)

Samples in 1% acetic acid were desalted and concentrated using C4-ziptips (Millipore, Billerica, MA), and analyzed in a Bruker REFLEX® II mass spectrometer in the positive ion mode using a matrix of α-Cyano-4-hydroxycinnamic acid (CHCA, Sigma). Spectra were analyzed by the open source software mMass 5.2.0.[48]

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

SDS-protein complexes were separated under reduced conditions on 8% polyacrylamide slab gels and stained with Gelcode Blue. Bands from PMPs charged with EnzB70k were excised from the gel, rinsed with 100 mM ammonium bicarbonate, and dried. As a control, the same segments were processed in parallel from lanes of material from PMPs charged with ChemBBSA. Cysteines were reduced using 10 mM dithiothreitol in 100 mM ammonium bicarbonate at 56°C and alkylated with 55 mM iodoacetamide. Proteins were digested overnight at 37°C with 12.5 ng/μL trypsin. The gel pieces were eluted four times, alternating between 50% acetonitrile, 5% formic acid and 50 mM sodium bicarbonate and an additional three times alternating between 100% acetonitrile and 50 mM sodium bicarbonate. Eluted peptides were combined and dried. Peptides were separated using a NanoAcquity ultra high-pressure liquid chromatography system (Waters) coupled to a LTQ Velos Pro or LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a precolumn (75 μm inner diameter, packed with Magic C18AQ 100 Å 5 μm particles, Bruker) at a flow rate of 1 μm min^{-1}. Peptides were eluted through an analytical column (75 μm inner diameter, packed with Magic C18AQ 100 Å 5 μm particles, Bruker) using a 90-min linear gradient from 7.5% to 30% acetonitrile with 0.2% formic acid and a flow rate of 250 nL min^{-1} with monitoring of effluent by data-dependent MS/MS.

For the LTQ Velos Pro, a survey scan performed in the ion trap (MS^1) was used to select ten precursors for tandem mass spectrometry (MS/MS) analysis. Selected peptides were fragmented by collision-activated dissociation (CAD, NCE = 35) and mass analyzed in the ion trap. A small window (±1.5 *m/z*) was used to exclude a maximum of 500 selected precursors for 30 s with a repeat count of 1. Automatic gain control was used at target values of 40,000 for MS^1 analysis and 40,000 for ion trap MS^2 analysis.

For the LTQ-Orbitrap survey scan performed in the Orbitrap (MS^1, resolving power = 60,000) was used to select eleven precursors for tandem mass spectrometry (MS^2) analysis. Selected peptides were fragmented by CAD (NCE = 35) and analyzed in the ion trap. Precursors with unassigned or +1 charge states were not selected for MS/MS analysis. Precursors were selected based on their calculated monoisotopic *m/z*. A small window (10 ppm) was used to exclude a maximum of 500 selected precursors for 90 s with a repeat

count of 1. Automatic gain control was used at target values of 1,000,000 for MS^1 analysis and 40,000 for ion trap MS^2 analysis.

MS/MS data were analyzed using the Coon OMSSA Proteomics Software Suite.[49] The Open Mass Spectrometry Search Algorithm was used to search spectra against a concatenated target-decoy database consisting of human protein sequences from Uniprot.[50] For all searches, tryptic peptides were created *in silico* allowing up to three missed cleavages. The precursor mass tolerance was set to ±5 Da for the LTQ Velos Pro and 100 ppm for the LTQ Orbitrap, and the monoisotopic mass tolerance was set to ±0.5 Da for fragments ions. Carbamidomethylation of cysteines was included as a fixed modification, and oxidation of methionines was added as a variable modification. Results from each experiment were then filtered to a 1% false discovery rate (FDR) using low resolution batch *FDROptimizer* for samples analyzed with the LTQ Velos Pro and high resolution batch *FDROptimizer* for the samples analyzed with the LTQ-Orbitrap Elite. The *FDROptimzer* conducts false discovery analysis at the peptide level using a two dimensional analysis. One of these dimensions is OMSSA e-value, whereas the other is the precursor mass error.

RESULTS

Previously, IFAST has been used to purify DNA, mRNA and proteins,[27-31] but this technology has yet to be tested for global identification of protein-protein interactions. We now have used IFAST to identify human plasma and platelet proteins that interact with 70k-Fn. Biotin-tagged 70k-Fn was bound to strepavidin-coated PMPs. In the initial development phase IFAST performance was characterized by detecting the interaction of the 70k-Fn bait protein with a bacterial peptide from *Strepcoccus pyogenes* known as functional upstream domain (FUD). FUD is a 56 amino acid peptide that binds strongly to 70k-Fn (Kd = 5.2 nM) by adding an extra β-strand to the existing β-sheet of multiple type I modules of Fn, a process known as β-strand addition.[36, 45] In addition, we characterized the d32-36 deletion mutant of FUD, which at 10 μM binds >20-fold less well to Fn than the 10 μM FUD as determined by a competition assay.[36] After this initial characterization, plasma and platelet proteins interacting with 70k-Fn were purified with IFAST and identified by LC-MS/MS.

We began by testing two techniques for inserting biotin into 70k-Fn. In one, the *N*-hydroxysulfosuccinimide (NHS) ester of biotin, which reacts with the primary amines of lysines, was inserted randomly at multiple sites within the protein. This method has the potential to disrupt the extended binding site available for β-strand addition. We therefore also explored the use of biotin protein ligase (BPL) that inserts biotin into a specific sequence motif.[12] One of these enzymes, BirA, modifies a short sequence[12, 23] that we grafted on to the C-terminus of recombinant 70k-Fn. To compare the interactions of FUD with 70k-Fn biotin tagged randomly at lysine residues with biotin-NHS (ChemB70k) or via the C-terminal BirA recognition sequence (EnzB70k), we utilized fluorescence polarization to quantify binding of FITC-FUD to the two proteins in solution. ChemB70k bound poorly whereas EnzB70k bound as strongly as underivatized 70k-Fn (Figure 1a). We then bound the two forms of 70k-Fn as well as ChemBBSA to PMPs and used fluorescence imaging to assess binding of FITC-labeled FUD after purification of the charged PMPs by IFAST. The EnzB70k-PMPs had much higher fluorescence in comparison to ChemBBSA-PMPs after purification with IFAST (Figure 1b – d) whereas the fluorescence of ChemB70k-PMPs was similar to the fluorescence of ChemBBSA-PMPs (not shown). As a third test of the importance of biotinylation, PMPs charged with the biotin-tagged 70k-Fn proteins were incubated with unlabeled FUD in TBS, and FUD associated with the PMPs after IFAST or a multi-wash purification was eluted with 1% acetic acid and detected by MALDI-TOF MS. Comparing the MALDI spectra from elutions of ChemB70k and EnzB70k, the peaks representing FUD were absent with ChemB70k, but were present from EnzB70k (Figure 1e

& f). The three types of experiments indicate that biotin-NHS inserts into the FUD-binding interface of 70k-Fn in a way that destroys the interface for β-zipper formation whereas EnzB70k has an intact binding site.

The MALDI-TOF mass spectra from elution of ChemBBSA-PMPs incubated with a mixture of FUD and the more weakly binding d32-36 derivative were measured after IFAST or a multi-wash protocol. ChemBBSA-PMPs washed a single time contained peaks representative of both polypeptides (not shown). These peaks disappeared only after three washes (not shown). In a parallel experiment, MALDI-TOF mass spectra of the elutions from IFAST purification did not contain signals for FUD or d32-36 when using ChemBBSA-PMPs (Figure 1g). The lack of FUD or d32-36 signal shows that a single pass of analyte through two oil barriers with IFAST removes non-binding components as efficiently as PMPs that have been washed manually three times.

Mass spectra from elutions of EnzB70k-PMPs contained peaks for FUD after one, two, and three steps of the manual wash purification. The spectral peaks representing d32-36 were present after one wash but were removed after two or three washes, indicating that the more weakly binding d32-36 was lost with the multi wash process (Figure 2a-c). In contrast, with IFAST, peaks for both FUD and the weaker binding d32-36 were identified after a single pass through IFAST (Figure 2d). The purification of FUD by both the manual wash protocol and IFAST using EnzB70k-PMPs but not with ChemBBSA-PMPs indicates the specificity of this interaction. Specific recovery of d32-36 peaks from EnzB70k-PMPs only with IFAST supports the superiority of IFAST in comparison to manual wash for affinity-based purification of weaker binding proteins. All MALDI-TOF mass spectra included a weak polymer signal at 2700 to 4200 m/z, due to leaching of polystyrene from PMPs used in the IFAST and manual wash purification. The polymer signal did not mask the FUD signal.

Interaction of EnzB70k with human serum proteins purified by IFAST

EnzB70k-PMPs and ChemBBSA-PMPs were incubated with plasma-serum prepared by clotting citrated plasma with thrombin. This fraction was selected because most of the fibrinogen, which is known to bind to 70k-Fn,[51] had been removed as fibrin, potentially freeing binding sites on EnzB70k-PMPs for other binding plasma proteins present. After purification of PMPs by IFAST, proteins bound to the PMPs and eluted with 1% acetic acid were separated by SDS-PAGE and detected by silver-staining. Bands were present in elutions of EnzB70k that were not present with the ChemBBSA-PMPs. (Figure 3b). To establish if these bands were due to inadequate wash steps, EnzB70k-PMPs incubated with serum were purified using an IFAST design containing one or two wash wells (diagrammed in Figure 3a). The additional wash wells did not affect the presence of these bands. Further, the addition of FUD blocked the binding of 70k-Fn to serum proteins, suggesting that specific binding is by β-zipper formation to the same extending interaction face. To appreciate the power of the purification, we compared SDS-PAGE patterns of proteins in the input and eluate wells (Supplementary Figure 1a). Lanes from the input wells were heavily overloaded with proteins whereas the lanes containing output proteins contained discrete bands, some of which were blocked with FUD.

Interaction of EnzB70k with human platelet proteins purified by IFAST

To identify interactions between 70k-Fn and platelet proteins, lysed platelets were incubated with either EnzB70k-PMPs or ChemBBSA-PMPs, purified with IFAST, and separated using SDS-PAGE electrophoresis. Specific reproducible bands at 45kDa, 70kDa, and 200kDa were seen on SDS-PAGE gels from elutions from samples containing EnzB70k, but not ChemBBSA-PMPs (Figure 3b). Similar to the results with serum, the presence of an additional wash well did not alter the pattern of binding between 70k-Fn and platelet

proteins (Figure 3b). Furthermore, the addition of FUD, modulated the binding of proteins present of platelet proteins with 70k-Fn. To judge further the specificity of the detected interactions between platelet proteins and EnzB70k, the loaded PMPs were incubated with 1μl, 5μl, or 10μl of platelet lysate. After purification with IFAST, the platelet lysate remaining post-purification within the input well and proteins eluted within 1% acetic acid were detected by SDS-PAGE and silver-stain (Supplementary Figure 1b). Comparisons of the bands in the elution and the input wells further confirm the specificity of the IFAST purification with EnzB70-PMPs.

nHPLC-MS/MS Analysis of specifically bound bands

The bands specifically identified by silver-staining (Figure 3 and Supplementary Figure 1) were located on SDS-PAGE stained with Gelcode Blue, excised from the gel, digested by trypsin and analyzed by nHPLC-MS/MS. To detect specific versus non-specific interaction, gel slices at the same locations were prepared from a matching gel of proteins eluted from ChemBSA-PMPs. Specific binding proteins were defined based on (i) peptides that were present from EnzB70k elutes and absent from ChemBBSA, or (ii) peptides with $\log_2$ ratio of peptide spectrum matches (PSMs) between bands excised from gels of proteins bound to EnzB70k and ChemBBSA of two or above or (iii) peptides with PSM $\log_2$ ratios of EnzB70k over ChemB70k above one that were present in two or more bands (for example actin). Proteins common to both samples were almost all derived from human epidermis.

Based on these criteria, 31 polypeptides from plasma-serum were identified as interacting with 70k-Fn and not with BSA (Table 1). Of these 31 polypeptides, 7 polypeptides are from 4 proteins known to interact with Fn: gelsolin;[52] B and C subunits of complement C1q;[53-56] α-, β -, and γ- chains of fibrin,[57] and Fn itself.[58] These results confirm the viability of the IFAST AP-MS approach for identifying the 70k-Fn interacting partners.

Similar inclusion and exclusion criteria were used to detect platelet proteins interacting with 70k-Fn (Table 2). Five polypeptides from platelet proteins were identified as interacting with 70k-Fn and not with BSA. The proteins identified include cytoplasmic actin, a protein known to interact with Fn,[59, 60] and myosin heavy chain-9.

Confirmation of nHPLC-MS/MS identifications by Western blotting

Western blot analysis of plasma-serum purified by IFAST detected gelsolin and inter-alpha-trypsin inhibitor heavy chain-2 (ITIH-2) in samples isolated with EnzB70k-PMPs, while gelsolin and ITIH-2 were not present in ChemBBSA-PMPs (Figure 4a&b). These interactions were blocked by FUD, further confirming results from silver-stained SDS-gels. Analysis by Western blot of platelet lysate purified with EnzB70k-PMPs detected two specific bands representative of myosin heavy chain-9 (Figure 4c).

DISCUSSION

Here we report the first exploratory use of IFAST affinity purification coupled with mass spectrometry for identification of protein-protein interactions. IFAST is a simple, easy-to-implement purification technique that is expandable to high throughput proteomic studies.[28, 31] Previously reported studies [28] and the current paper indicate the superiority of IFAST versus multi step wash purification for detecting weaker binding proteins (Figure 2). The enhanced purification of weak binding proteins is due to the almost instantaneous separation of bound and free protein. Protein-protein interactions at equilibrium are defined by the rates of association and dissociation. The short time in the wash well preserves interactions for which the dissociation constant is rapid. Thus, The MALDI-TOF MS spectra from elutions of EnzB70k-PMPs purified with IFAST or multi step wash contained the

strong binding peptide FUD, whereas only spectra from IFAST purifications contained signals for both FUD and d32-36.

To control for non-specific protein-protein interactions, all experiments included a parallel separation with ChemBBSA-PMPs. Comparisons of MALDI spectra and silver-stained SDS-PAGE from EnzB70k-PMPs and ChemBBSA-PMPs indicates the specificity of the detected signals and bands. To confirm further the specificity of bands present on SDS-PAGE and gain insight into the mode of binding, a second control was done by including FUD, which binds to 70k-Fn by β-strand addition, in the incubations. [36]

The plasma-serum proteins interacting with 70k-Fn included seven polypeptides from four different proteins that are known to interact with Fn. Gelsolin is both cellular and plasma protein; the cellular form modulates actin polymerization whereas plasma gelsolin has been suggested to assist in clearance of actin from the circulation.[52, 61, 62] The presence of gelsolin was confirmed by Western blot. Our results narrow down the binding region in Fn to the 70k region and further demonstrate that this interaction is blocked by FUD, suggesting that the binding site for gelsolin on Fn overlaps with the binding site for FUD. The EnzB70k-PMP eluate was enriched in B and C subunits of complement protein C1q. C1q contains a collagen-like region that enables its interaction with complement C1r and fibronectin.[53, 54] The collagen-binding site of Fn is known to be in the 70k region.[42] The C1q-fibronectin interaction has been suggested to be important in reticuloendothelial clearance of C1q-coated immune complexes.[55, 56] Other identified proteins known to interacting with Fn included α-, β-, and γ- chains of fibrin(ogen), full-length Fn (~220kDa) and 70k-Fn.[57, 58] Fibrin(ogen) is known to interact with Fn through regions within 70k-Fn.[57] Finally, full-length Fn is known to interact with itself using the 70k-Fn region.[58]

In addition to the known binding proteins in plasma-serum, 24 polypeptides of proteins previously not known to interact with 70k-Fn were purified with IFAST and identified by nHPLC-MS/MS. Additional experiments using these proteins in a purified form will be needed to learn if the isolation was due to direct interaction with 70k-Fn or by indirect interaction through formation of complexes with the other interactors of 70k-Fn. Prothrombin was identified based on peptides with sequence matches from all regions within the protein with the exception of the Gla region (data not shown), indicating isolation of the prothrombin zymogen rather than active thrombin that was bound to fibrin(ogen). Additional complement proteins, factor B, C1s, C3, C4, C6, and C9, were isolated. One or more of these proteins may be present due to their interaction with complement C1q, but further studies are required to confirm this hypothesis.[63] We identified several members of the inter-alpha-trypsin inhibitor family (IαI). This family of proteins is composed of a common light chain called bikunin, bound to closely related heavy chains.[64] nHPLC-MS/MS data indicated the presence of IαI heavy chains ITIH-1, ITIH-2, and ITIH-4. The presence of ITIH-2 was confirmed by Western blot. Interestingly, this interaction was blocked by FUD, suggesting that the binding site for ITIH-2 overlaps with the binding site for FUD. All known heavy chains contain Von Willebrand factor-A (vWA) domains that are present in many species of extracellular matrix proteins and play an important role in formation of protein complexes.[65] Complement factor B also contains vWA domains that can enable interaction with IαI heavy chains.[66, 67] However, pull-down and functional experiments have identified interaction of ITIHs with complement C3 and C4 and implicated IαIs in modulating the protease activity of the complement C3C4 complex.[68] Therefore, IαI member proteins may be interacting with 70k-Fn through complex formation with complement proteins or vice versa, i.e., some of the complement proteins may interact with Fn by IαIs. An additional explanation for the presence of IαI heavy chains is interaction of 70kD-Fn with hyuloronan, which is a known interactor with Fn and is covalently linked to IαI heavy chains.[64, 69] In addition to members of IαI family of protease

inhibitors, five members of the serine protease inhibitor (SERPIN) family, alpha1-anti-trypsin, alpha2-antiplasmin, kallistatin, thyroxine binding globulin, and corticosteroid binding globulin, were found to interact preferentially with EnzB70k-PMPs.[70]

Platelet proteins interacting with 70k-Fn included actin, which is known to interact with Fn by the 70k-Fn region.[59, 60] Actin, however, represented the most abundant band in gels of whole lysates (Supplementary Figure 1b), and PSMs for actin were common in control slices. In contrast, many more peptides from myosin heavy chain-9 were detected from eluates of EnzB70k than of ChemBBSA, and these peptides were found in two bands, one of 190 kD that contained PSMs from throughout the protein and a second of 130 kD that contained peptides from 746-1960 (Supplementary Figure 2). The sequence found in both bands constitutes the rod domain, which forms an α-helical coiled-coil.[71] The presence of intact and fragmented myosin heavy chain-9 was confirmed by Western blot. The binding of myosin heavy chain-9 was blocked with FUD, suggesting similar binding regions within 70k-Fn for both polypeptides (Figure 4) and raising the question of the conformation adopted by myosin as it binds to 70k-Fn. The 130 kD fragment is similar to in size to the well-known heavy meromyosin rod fragment of skeletal muscle myosin.[72] The presence of a prominent 130 kD fragment within the EnzB70k elutions and its absence from input lysate suggests that either binding of myosin to 70k-Fn labilizes myosin to limited fragmentation by a protease present in the platelet lysate or already formed rod-like domain of platelet myosin binds more strongly to 70k-Fn than intact myosin and is selectively bound from the lysate.

Many of the other interactors detected from plasma-serum or platelet lysate were present at several molecular weight positions both larger and smaller than the expected full-length polypeptide. Differences resulting in greater than expected masses could arise from amide cross-links introduced by transglutaminases, or other covalent linkages or adducts. Proteolysis presumably accounts for the smaller molecular weight bands. The limit of detection for silver staining is ~250 fmols of average-sized protein, which is three orders of magnitude less sensitive than 1-attomol detection limit for LC/MS/MS. [73] Thus, the proteins of unexpected size detected by LC/MS/MS may represent small percentages of the forms of the protein that were isolated by IFAST.

The results presented in this paper validate IFAST coupled with MS as a tool for proteome research. AP-MS is a powerful tool for detecting protein-protein interactions and characterizing the interactome. Current AP-MS approaches are limited by affinity purification approaches that remove weak binding protein. IFAST can reduce this loss of weak interacting proteins. In addition to better purification performance, IFAST requires small sample volumes and is amenable to high-throughput proteomic studies. Furthermore, the use of PMPs armed with a predetermined density of bait proteins or exact control of interaction time could enable quantification of competition among interacting proteins for the bait and kinetics of the interaction. Finally, IFAST allows facile analysis of proteins remaining in the input sample after purification, and could be extended to affinity purifications using two or more bait proteins. Our validation included an initial characterization using FUD followed by use of IFAST to isolate interactors from two biological samples with known interactors. We identified both known and previously unidentified interactors. The 70k N-terminus of fibronectin is known to regulate fibronectin fibril formation, which is important in many physiologic and pathologic processes,[36, 37] but little is known about proteins that interact with 70k-Fn. Several proteins implicated in assembly, including integrins and transglutaminase-2,[58] were not encountered. However, further isolations sampling a variety of interaction conditions, which can be accomplished easily with IFAST, may reveal such proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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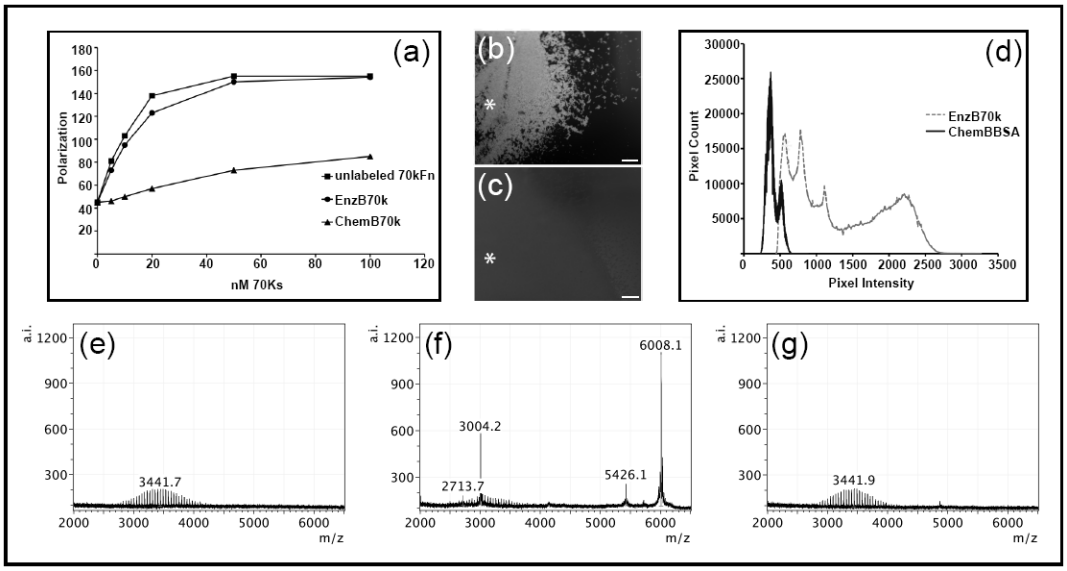

Figure 1.

Comparison of biotin-labeled proteins and the interaction with FUD (a) Binding of FITC-FUD to unlabeled 70kFn (■), EnzB70k (●) and ChemB70k (▲). FITC-FUD, 20 nM, was mixed with equal volume of unlabeled 70K, EnzB70K and ChemB70K 5, 10, 20, 50, or 100nM. After a 2-hr incubation, fluorescence polarization of FITC-FUD in each group was monitored. (b and c) Fluorescent microscopic images of FITC-FUD associated with PMPs charged with (b) EnzB70k or (c) ChemBBSA. Beads (asterisks) were collected at one side of the well with a magnet. (d) Fluorescence intensity histogram of fluorescence imaged in panels b and c. (e-g) MALDI spectra from peptide mix of FUD (Avg[H^+] = 6007.4) and d32-36 (Avg[H^+] = 5425.3) purified with IFAST using PMPs charged with (e) ChemB70k, (f) EnzB70k, (g) ChemBBSA. EnzB70k has MS signals for FUD and d32-36, while ChemB70k and ChemBBSA have no signal. There is polymer signal between 2600 to 4300 m/z (mMass 5.2.0). White bars indicate 100 μm.

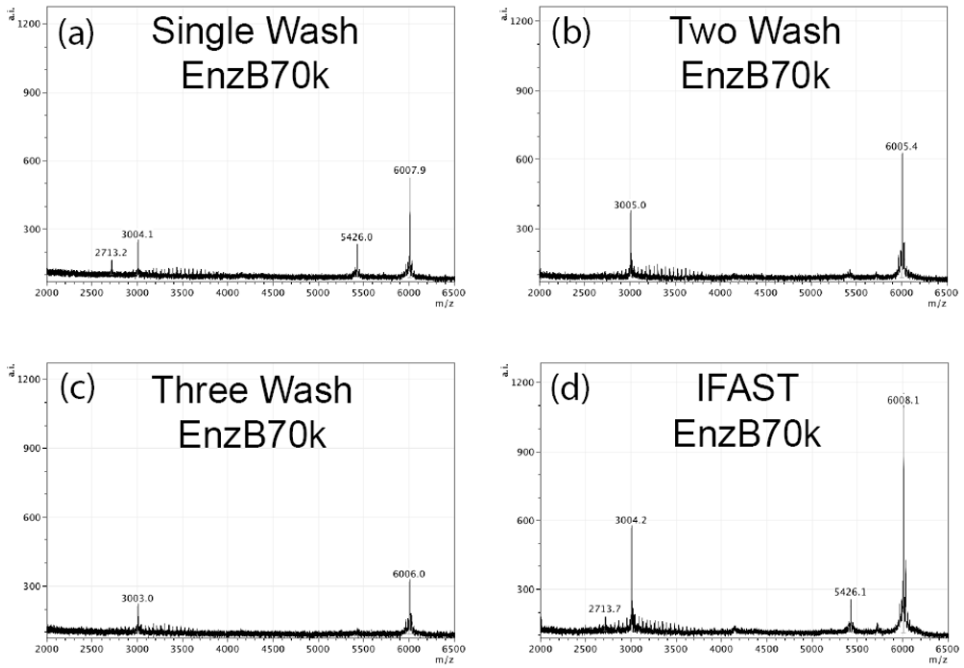

Figure 2.

IFAST versus wash purification from elutions of EnzB70k-PMPs after incubation with a peptide mix of FUD (Avg[H^+] = 6007.4) and d32-36 (Avg[H^+] = 5425.3). Both IFAST and wash purifications contained peaks for the strong binding FUD. Wash purification, spectral peaks representing d32-36 were present after (a) one wash, but were removed after (b) two or (c) three washes. (d) Mass spectra from elutions purified with IFAST contained peaks for both FUD and d32-36.

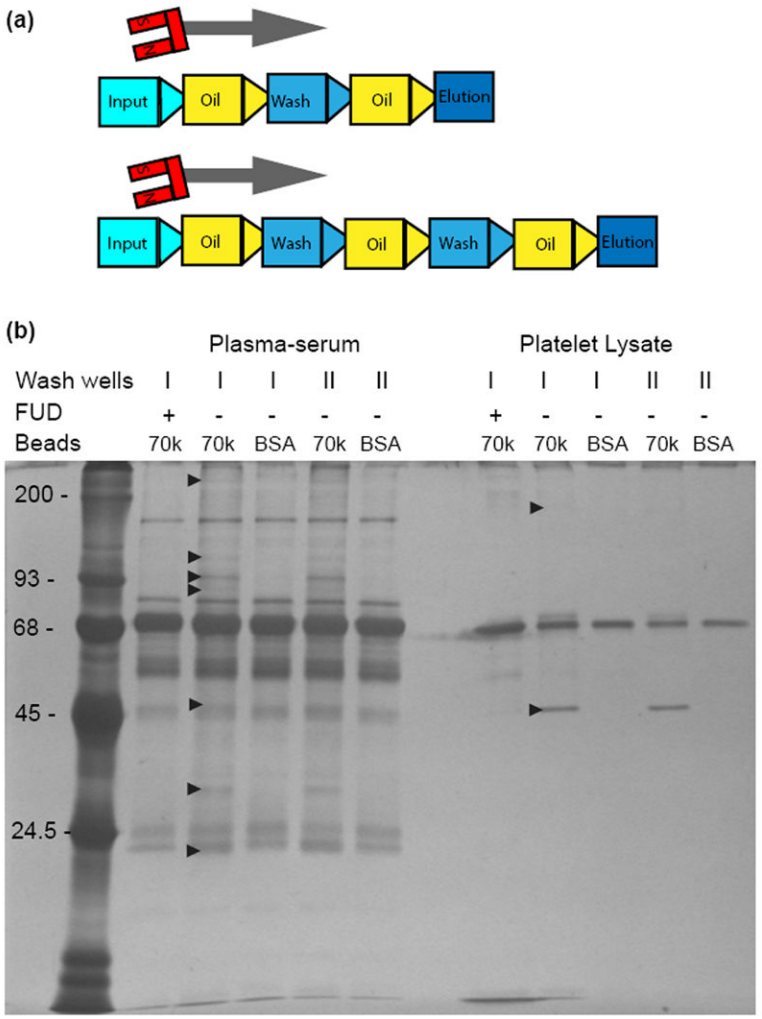

Figure 3.

Isolation of specific plasma-serum and platelet lysate proteins with EnzB70k using IFAST (a) Diagram illustrating the difference between one- and two-wash IFAST. (b) Proteins from plasma-serum or platelet lysate were purified with EnzB70k- or ChemBBSA-PMPs using IFAST. To characterize the role of multiple wash wells on IFAST purification, one wash or two wash IFAST devices were used for the purification. Purified proteins were analyzed by SDS-PAGE followed by silver-staining. Lanes are labeled according to the sample being analyzed, number of wash wells, presence (+) or absence (-) of FUD, and the bait protein bound to PMPs. Arrowheads indicate the bands that were excised in a similar gel stained with Gelcode blue and labeled to correspond to Tables 1 and 2.

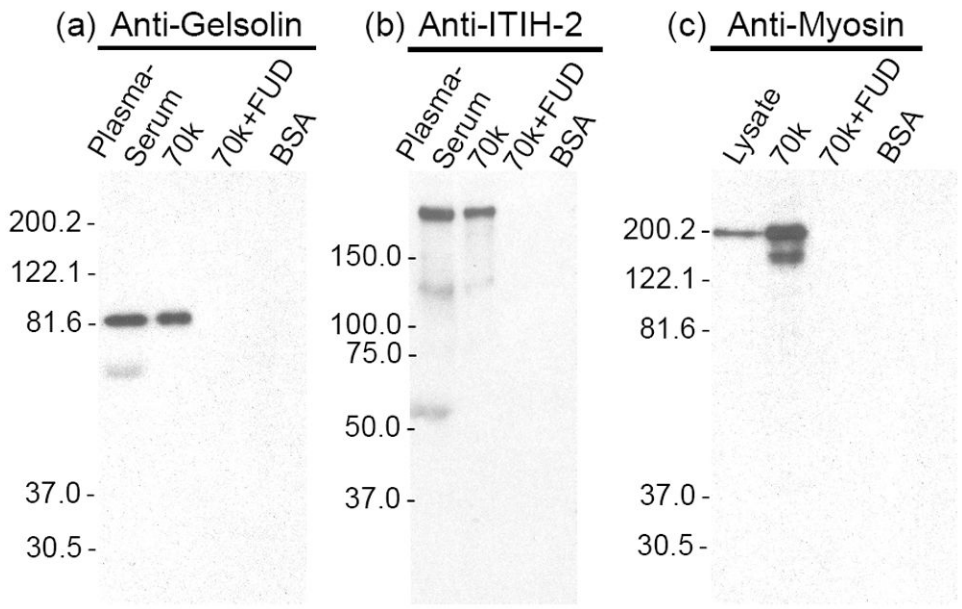

Figure 4.

Biochemical confirmation of protein hits from mass spectrometry. Biochemical confirmation of protein hits from mass spectrometry. (a) Anti-gelsolin Western blot of plasma-serum proteins purified by IFAST with EnzB70k-PMPs or ChemBBSA-PMPs. The interaction between gelsolin and EnzB70k-PMPs, was specific and blocked with FUD. (b) Anti-inter α trypsin inhibitor heavy chain-2 (ITIH-2) Western blot of plasma-serum proteins purified by IFAST with EnzB70k-PMPs and ChemBBSA-PMPs. The interaction between ITIH-2 and EnzB70k-PMPs, was specific and blocked with FUD. (c) Anti-myosin heavy chain-9 Western blot of platelet lysate proteins purified by IFAST with EnzB70k-PMPs or ChemBBSA-PMPs. The interaction between myosin heavy chain-9 and EnzB70k-PMPs was specific and blocked with FUD. The following were analyzed in each set of 4 lanes: 0.1 μl (with the exception of the ITIH-2 blot which contained 0.5 μl) plasma-serum or lysate representing 1% (5% for the ITIH-2 blot) of what was added to the input wells and all of what bound to EnzB70k, EnzB70k in the presence of FUD, or ChemBBSA.

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Table 1

List of plasma-serum proteins interacting with 70k-Fn and detected by nHPLC-MS/MS.

M.W. (kDa)	UniProt Code	Abbreviation	Protein Description	# AA	% Sqn	PSMx/PSMc	PSM Ratio
240	**P02751-15**	**FINC_HUMAN**	**Fibronectin Full Length**	2477	6.30	10/0	3.32
	E7EVA3	E7EVA3_HUMAN	Complement factor B	1266	3.95	5/0	2.32
	P06396	**GELS_HUMAN**	**Gelsolin**	782	9.46	4/0	2.00
	P09211	GSTP1_HUMAN	Glutathione S-transferase	210	27.14	4/0	2.00
	P02746	**C1QB_HUMAN**	**Complement C1q subcomponent subunit B**	253	11.46	2/0	1.00
	P02747	**C1QC_HUMAN**	**Complement C1q subcomponent subunit C**	253	17.55	4/0	2.00
	B0UZ83	B0UZ83_HUMAN	Complement C4 gamma chain	1744	1.55	2/0	1.00
	P31944	CASPE_HUMAN	Caspase-14	242	11.57	3/0	1.58
	P19827	ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	911	22.94	19/7	1.44
	P19823	ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2	946	25.48	18/9	1.00
	B7ZKJ8	B7ZKJ8_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	935	2.25	2/0	1.00
	P00734	THRB_HUMAN	Prothrombin	622	3.54	2/0	1.00
120	P13671	CO6_HUMAN	Complement component C6	934	7.07	5/0	2.32
	P19827	ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	911	2.42	2/0	1.00
	P19823	ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2	946	6.87	5/0	2.32
	B7ZKJ8	B7ZKJ8_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	935	29.2	19/4	2.25
100	B4E1Z4	B4E1Z4_HUMAN	Complement factor B	1266	8.37	9/3	1.58
	P09871	C1S_HUMAN	Complement C1s subcomponent	688	9.16	5/0	2.32
	P06396	**GELS_HUMAN**	**Gelsolin**	782	5.50	4/0	2.00
	B7ZKJ8	B7ZKJ8_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	935	5.46	4/1	2
	C9JC841	C9JC84_HUMAN	Fibrinogen gamma chain	461	4.77	2/0	1.00
93	**P06396**	**GELS_HUMAN**	**Gelsolin**	782	26.73	20/0	4.32
	E7EVA3	E7EVA3_HUMAN	Complement factor B	1266	3.87	3/0	1.58
	P09871	C1S_HUMAN	Complement C1s subcomponent	688	4.51	2/0	1.00
	P01024	CO3_HUMAN	Complement C3	1633	3.13	3/0	1.58
	P00734	THRB_HUMAN	Prothrombin	622	8.68	4/0	2.00
80	P00734	THRB_HUMAN	Prothrombin	622	25.72	15/0	3.91

M.W. (kDa)	UniProt Code	Abbreviation	Protein Description	# AA	% Sqn	PSMx/PSMc	PSM Ratio
	P02787	TRFE_HUMAN	Serotranferrin	698	3.30	2/0	1.00
60	**P02675**	**FIBB_HUMAN**	**Fibrinogen beta chain**	491	54.38	27/0	4.75
	P00734	THRB_HUMAN	Prothrombin	622	32.96	15/0	3.91
	P19827	ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	911	4.39	3/0	1.58
	P19823	ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2	946	13.11	12/0	3.58
	P02751-15	**FINC_HUMAN**	**Fibronectin 70kDa**	2477	4.04	9/0	3.17
	P02671	**FIBA_HUMAN**	**Fibrinogen alpha chain**	866	11.09	9/0	3.17
	C9JC84	**C9JC84_HUMAN**	**Fibrinogen gamma chain**	461	11.50	4/0	2.00
	E7EVA3	E7EVA3_HUMAN	Complement factor B	1266	5.06	6/0	2.58
	P02747	**C1QC_HUMAN**	**Complement C1q subcomponent subunit C**	247	12.24	2/0	1.00
	P09871	C1S_HUMAN	Complement C1s subcomponent	688	3.63	2/0	1.00
	P01024	CO3_HUMAN	Complement C3	1633	7.82	11/2	2.46
	B0UZ83	B0UZ83_HUMAN	Complement C4 gamma chain	1744	2.75	4/1	2.00
	P04003	C4BPA_HUMAN	C4b-binding protein alpha chain	597	3.85	2/0	1.00
	P02748	CO9_HUMAN	Complement component C9	559	5.55	3/0	1.58
	P29622	KAIN_HUMAN	Kallistatin	427	12.41	5/0	2.32
	sP01781	HV320_HUMAN	Ig heavy chain V-III GAL	117	18.52	2/0	1.00
	P05543	THBG_HUMAN	Thyroxine-binding globulin	415	5.06	2/0	1.00
	Q96IY4	CBPB2_HUMAN	Carboxypeptidase B2	423	6.38	2/0	1.00
	P08185	CBG_HUMAN	Corticosteroid-binding globulin	405	5.68	2/0	1.00
	P08697	A2AP_HUMAN	Alpha-2-antiplasmin	491	4.89	2/0	1.00
30			Bovine albumin / Human albumin				
20	**P02747**	**C1QC_HUMAN**	**Complement C1q subcomponent subunit C**	247	20.41	5/0	2.32
	P02751-15	**FINC_HUMAN**	**Fibronectin**	2477	2.22	4/0	2.00
	B9A064	IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5	214	23.36	4/0	2.00
	P01009	A1AT_HUMAN	Alpha-1-antitrypsin	418	8.37	4/1	2.00
	P19823	ITIH2_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H2	946	4.65	3/0	1.58
	P02671	FIBA_HUMAN	Fibrinogen alpha chain	866	3.12	2/0	1.00
	P18136	KV313_HUMAN	Ig kappa chain V-III region HIC	129	19.38	2/0	1.00
	P02787	TRFE_HUMAN	Serotransferrin	698	2.87	2/0	1.00

Proteins were selected by comparing tryptic peptide sequence matches (PSM) for elutions from EnzB70k (experimental) to ChemBBSA (control). Proteins known to interact with Fn are in bold. Peptide sequence matches for Fn from the excised band at 240 kDa covered regions present within full-length Fn, while sequence matches from the band at around 60 kDa covered only regions present within 70-Fn (PSMx: # experimental peptide sequence matches, PSMc: # control peptide sequence matches for BSA, # AA: total number of amino acids in the protein, %Sqn: percent sequence coverage by the experimental PSM, PSM ratio is the log2 of PSMx/PSMc where PSMc is set to 1 in the case of PSMc equal to 0).

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Table 2

List of platelet proteins interacting with 70k-Fn and detected by nHPLC-MS/MS.

M.W. (kDa)	UnitProt Code	Abbreviation	Protein Description	# AA	% Sqn	PSMx/PSMc	PSM Ratio
190	P35579	MYH9_HUMAN	Myosin-9	1960	38.01	89/3	4.89
	I3L3I4	**I3L3I4_HUMAN**	**Actin; cytoplasmic 2**	383	12.27	4/2	1.00
130	P35579	MYH9_HUMAN	Myosin-9	1960	7.09	12/0	3.58
	P01009	A1AT_HUMAN	Alpha-1-anti-trypsin	418	14.35	4/0	1.00
	I3L3I4	**I3L3I4_HUMAN**	**Actin; cytoplasmic 2**	383	12.79	4/0	1.00
70	**P02751-15**	**FINC_HUMAN**	**Fibronectin 70kDa**	2477	8.96	28/1	4.81
	P04406	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	335	8.36	2/0	1.00
	I3L3I4	**I3L3I4_HUMAN**	**Actin; cytoplasmic 2**	383	5.22	2/0	1.00
45	**I3L4N8**	**I3L4N8_HUMAN**	**Actin; cytoplasmic 2**	242	45.87	6/2	1.58
	F5H0N0	**F5H0N0_HUMAN**	**Actin; cytoplasmic 2**	333	27.03	10/5	1.00

Proteins were selected by comparing tryptic peptide sequence matches (PSM) for elutions from EnzB70k (experimental) to ChemBBSA (control). Proteins known to interact with Fn are in bold. Peptide sequence matches for Fn were only present in the 70kDa excised gel, and the sequence matches were only from the 70k-Fn region, suggesting some release of EnzB70k from the PMPs within the elution (PSMx: # experimental peptide sequence matches, PSMc: # control peptide sequence matches for BSA, # AA: total number of amino acids in the protein, %Sqn: percent sequence coverage by the experimental PSM, PSM ratio is the log2 of PSMx/PSMc where PSMc is set to 1 in the case of PSMc equal to 0).