Supporting Information

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SI Materials and Methods

Isolation and Activation of *Ascaris suum* Sperm. *Ascaris* males were collected from the intestines of infected hogs at Zhongrui Pork Processors and Jinluo Meat Products. They were placed in worm buffer [PBS containing 100 mM $NaHCO_3$ (pH 7.0) 37 °C] and transported to the laboratory and stored at 37 °C overnight for recovery. Spermatids were obtained by dissecting males, removing the seminal vesicle, and extruding the seminal fluid into HKB buffer [50 mM Hepes, 70 mM KCl, 10 mM $NaHCO_3$ (pH 7.1)]. Spermatids were activated to extend pseudopods in HKB buffer with the addition of a glandular vas deferens extract (SAS) (1). Live cells were pipetted into chambers formed by mounting a 22 × 22-mm glass coverslip onto a glass slide with two parallel strips of two-sided tape and examined with an Axio Imager M2 microscope (Carl Zeiss) equipped with a 40 × differential interference contrast (DIC) objective lens with appropriate filters. Images were captured with a charge-coupled device (CCD) (Andor) and processed with MetaMorph software (Universal Imaging). Sperm not used immediately were pelleted at 10,000 *g* for 30 s, and the cell pellet was stored at −80 °C after the removal of supernatant.

Preparation of Sperm Extracts (S100) and Fiber Assembly. Frozen spermatozoa were thawed on ice for 1 h and subject to centrifugation at 16,000 × *g* for 10 min. The supernatant was subject to centrifugation at 100,000 *g* for 1 h at 4 °C. The supernatant (S100) was used for the fiber assembly assay with the addition of 1 mM ATP in KPM buffer (10 mM potassium phosphate, 0.5 mM $MgCl_2$, pH 6.8) (2).

Purification of the 46-kDa Protein Recognized by the 1CB4 Antibody from Sperm Extracts. The sperm extract was diluted 1:4 with KPM buffer and fractionated by ammonium sulfate precipitation (3). Each pellet was resuspended in KPM buffer and dialyzed against KPM buffer for 24 h. The different fractions were analyzed using SDS/PAGE and Western blots with the 1CB4 antibody. The protein recognized by 1CB4 (~46 kDa) was enriched in the 60–80% fraction of ammonium sulfate ($AmSO_4$), and this fraction was dialyzed against pH 7.8 KPM buffer and fractionated in 1 mL SP-Sepharose High Performance cation exchange columns (GE Healthcare) using an AKTA FPLC system (GE Healthcare). The fraction containing the protein recognized by 1CB4 was loaded onto a Heparin HP affinity chromatography column (GE Healthcare) and eluted with a 0–500 mM NaCl gradient in 10 mM KPM (pH 7.8). The eluate contained a single 46 kDa band, as resolved by SDS/PAGE.

Immunodepletion Assay. The As_SRP-1 polyclonal antibody (10 μg/mL) was bound to Protein A Sepharose 4B (GE Healthcare), and these were washed five times with HKB buffer. Washed beads were then incubated with As_SRP-1 (10 μg/mL) 4 °C for 1 h to immobilize As_SRP-1 onto the beads. After SAS (0.5 μg/mL) was added to the preloaded beads for 1 h with rotation, the supernatant was collected to examine its effect on sperm activation. For mock depletion, either As_SRP-1 antibody was replaced with IgG or As_SRP-1 was omitted for binding with beads before incubation with SAS.

Purification of Sperm Activator As_TRY-5 from SAS. The SAS was separated by $AmSO_4$ precipitation. The 40–60% cut fraction was loaded onto a HiTrap phenyl HP column (GE Healthcare; 5 mL) equilibrated with HKB buffer [50 mM Hepes, 70 mM KCl, 10 mM $NaHCO_3$ (pH 7.1); all of the columns mentioned below had been equilibrated with HKB buffer] containing 1 M $AmSO_4$, then this fraction was sequentially eluted with HKB buffer containing 0.5 M $AmSO_4$ (50% eluate) or 0 M $AmSO_4$ (100% eluate), respectively. The 100% eluate contained sperm-activating activity, which was concentrated with an Amicon Ultra-4 centrifugal filter devices (10 K cutoff) and loaded onto a Superdex 200 column (GE Healthcare; 120 mL). The fractions containing the sperm-activating activity were pooled and loaded onto a HiTrap Q HP column (GE Healthcare; 5 mL), which was eluted with a 60 mL linear gradient of HKB buffer containing 0–0.8 M NaCl. The peak fractions were again pooled, concentrated and loaded onto a Mono Q 5/50 GL column (GE Healthcare; 1 mL), and the column was eluted with a 60 mL linear gradient of HKB buffer containing 0.2–0.8 M NaCl. The sperm-activating peak fractions eluted with 0.6–0.8 M NaCl. Therefore, these fractions were pooled, concentrated with an Amicon Ultra-4 centrifugal filter devices (10 K cutoff) and loaded onto a HiTrap Con A 4B columns (GE Healthcare; 1 mL), following by the elution with HKB buffer containing 0.5 M methyl-α-d-glucopyranoside. Finally, the eluate was concentrated and incubated with purified As_SRP-1 at 4 °C for 12 h. The incubated mixture showed a new shifted ~90-kDa band by SDS/PAGE, and this band was recognized by our anti-As_SRP-1 antibody in Western blots. The 90-kDa band was excised from the gel for MS analysis and de novo sequencing.

MS Identification of As_SRP-1 As the 1CB4-Recognizing Protein. ***MS data collection for de novo sequencing.*** A highly purified *Ascaris* protein sample recognized by the 1CB4 antibody consisted of three closely migrating proteins around 46 kDa. This sample was digested with trypsin, loaded onto a C18 reverse-phase column with a pulled tip (100 μm ID; 8 cm in length), and packed with 3-μm, 125-Å Aqua C18 resin (Phenomenex). LC-MS/MS analysis was performed over a 70-min run on an LTQ-Orbitrap-ETD mass spectrometer (ThermoFisher Scientific) connected to an Agilent 1200 quaternary HPLC Pump. The HPLC gradient was made of buffer A (acetonitrile/H_2O/formic acid, 5/95/0.1) and buffer B (acetonitrile/H_2O/formic acid; 80/20/0.1) at a constant flow rate of 0.1 mL/min. The flow was split between a waste line and the C18 column. The resulting flow rate at the tip of the column was about 200 nL/min, and the gradient was as follows: 0 min, A = 100.0%; 2 min, A = 90.0% B = 10.0%; 32 min, A = 50.0% B = 50.0%; 42 min, B = 100.0%; 47 min, B = 100.0%; 50 min, A = 100.0%; 70 min, A = 100.0%. Three sets of higher-energy collisional dissociation (HCD) data were generated and in each, full scans (R = 30,000) and data-dependent HCD (normalized collisional energy = 40%) MS2 scans (R = 7,500) were all analyzed in the Orbitrap. For dataset 1 (HCD only), the top 5 most intense +2 precursor ions detected in each full scan were isolated for MS2 using HCD. Dynamic exclusion was turned on with two repeat counts, 5 s repeat duration, exclusion list 500, and exclusion duration 15 s. For dataset 2 (normal-mass range HCD), only the most intense +2 or +3 precursor ion in each full scan was isolated to generate four MS2 spectra: HCD (mass range100–2,000 m/z), CID in LTQ, high-resolution electron transfer dissociation (ETD) spectra mass analyzed in the Orbitrap, and ETD in LTQ. The dynamic exclusion was set at repeat count 1, exclusion list 500, and exclusion duration 10 s. For dataset 3 (low-mass range HCD), the two most intense +2 or +3 precursor ions in each full scan were isolated and for each four MS2 spectra were generated: HCD (mass range 50–2,000 m/z), CID in LTQ, high-resolution ETD (detected in the Orbitrap), and ETD in LTQ. The dynamic exclusion setting was the same as in dataset 2 except that exclusion duration was 15 s All tandem mass spectra were extracted from RAW files using Xcalibur 2.0.7. Different types of MS2 spectra were separated by an in-house software

MS2Extractor. Only the HCD and high-resolution ETD data were used in de novo peptide sequencing analysis.

Identification of As_SRP-1 by de novo sequencing. Peptide sequences were deduced from normal- and low-mass range HCD spectra, as well as high-resolution ETD spectra using a de novo sequencing program called pNovo (4). The de novo sequencing results were pooled together, from which redundant peptide sequences and those with less than six amino acids or a *C*-score less than 0.5 were removed. The resulting 425 unique peptide sequences were BLAST searched against an *Ascaris* protein database translated from EST sequences (ASP_NEMBASE3_pro.fsa from www.nematodes.org). The PAM30 matrix, a word length of 2 aa and an E-value cutoff of 20,000 were used for BLASTP search. The BLAST results were tabulated and filtered, requiring >50% coverage (length of matched sequence/length of query peptide sequence) and >60% identity (number of identical amino acids within the matched sequence/length of matched sequence) for each retrieved match. For each protein found by BLAST search, the total number of unique peptide matches was calculated. With 60 unique peptide hits and 173 HCD spectra, As_SRP-1 ascended to the top. This was followed by a copurified contaminant CTS-1 (citrate synthase) with 29 unique peptide hits obtained from 85 spectra.

Identification of As_TRY-5 by de Novo Sequencing. The 90-kDa covalent complex between As_SRP-1 and its target protease was excised from the SDS/PAGE gel, in-gel digested with trypsin, Asp-N, and Lys-N, separately. LC-MS/MS analyses of the resulting peptides were the same as described above except for the following changes in MS data collection. Each MS (R = 30,000) was followed by 10 MS2 on top 2 ions with HCD (100–2,000 m/z), low-mass range HCD (50–2,000 m/z), CID in LTQ, high-resolution ETD in Orbitrap, and ETD in LTQ for each ion, R = 7500 for HCD and high-resolution ETD. We used pNovo to identify peptides from the HCD and high-resolution ETD spectra of the 90 kDa band. Sequences belonging to As_SRP-1 and contaminant proteins HSP90, keratins, trypsin, Lys-N, or Asp-N (these proteins were found in the sample by database search) were removed. Overlapping peptides found by pNovo were assembled into longer sequences using an in-house script. BLAST search matched these contiguous sequences to trypsin-like protease protein 5 [*Brugiya malayi*] with an E value as low as 8e-08. Its closest homolog in *C. elegans* is TRY-5. Thus, the protease target of As_SRP-1 is named As_TRY-5 in *Ascaris suum*.

Molecular Cloning of *As_srp-1* and *As_try-5*. Total RNAs from *Ascaris* testes and vas deferens were prepared using TRIzol reagent (Invitrogen) and the poly(A)$^+$ RNA was purified using Oligotex (QIAGEN). The first-strand cDNA was produced using a FirstChoice RLM-RACE kit (Ambion). For the 5′ RACE PCR template, the 5′ adaptor provided in the kit was ligated to the CIP/TAP-treated RNA using T4 RNA ligase and the reverse transcription was performed using random decamers as primers. For the 3′ RACE PCR template, reverse transcription was performed using a 3′ adaptor as primer.

To obtain the full-length *As_srp-1*, the 5′ RACE primers (P1: CATCATATCGACCATTATGCGTTG; P2: TGGATATTGCCAACTTCCATTGA) and 3′ RACE primers (P3: GTGGCATTTGTGCAAGACCAACAC; P4: ATTCGTCGTGCGTTCAGCATGAC) were designed according to the *Ascaris* EST database. PCR amplification was performed with *PfuUltra* II fusion HS DNA polymerase (Stratagene) and the PCR products were cloned into *pEASY*-Blunt vectors (TransGen) for sequencing.

For *As_try-5* cloning, first we performed 3′ RACE PCR using degenerative primers (2F: GGATCCGAYGARTTYGAYGARTGGGA; and 7F: GGATCCGGNGTNTGYGAYGAYGARGA) designed according to peptides deduced from de novo sequencing and obtained a 406 bp *C*-terminal sequence. The underlined sequences in primers show the restriction site for increasing the melting temperature and specificity when the PCR was performed. Subsequently, 5′ RACE PCR was performed using primers R1 (CAATCACCCAAACACAACAACAAG), R2 (TTTGTCAGTTTTTTGTTTCCTCCAC), R3 (GTTATGAGTGTTCCTCCGCAGTCG), and R4 (TGTTCCTCCGCAGTCGGCTTCTAA), and the PCR products were cloned into *pEASY*-Blunt vector (TransGen) for sequencing.

Recombinant As_SRP-1 Expression in *E. coli*. The ORF of *As_srp-1* was amplified from cDNA and cloned into pET-28a vector (Novagen). The resulting plasmid was transformed into *E. coli* strain BL21 (DE3) for expressing recombinant As_SRP-1. *E. coli* lysates were analyzed by SDS/PAGE and Western blotting with either a polyclonal antibody against As_SRP-1 (anti-P46) or the monoclonal 1CB4 to test the de novo sequencing result.

Production of a Polyclonal Antibody Against As_SRP-1. To generate a polyclonal antiserum against As_SRP-1, we injected 0.5 mg As_SRP-1 purified from sperm extract (as described above) into a New Zealand white rabbit, and the rabbit was immunized five times. One week after the last injection, the rabbit was killed and serum was collected. The antibody was then purified from the antiserum using protein A-Sepharose HP columns (GE Healthcare).

Immunofluorescence Assay. Spermatids, spermatozoa, or fibers grown in vitro were fixed with 1.25% glutaraldehyde in buffer (HKB buffer for sperm and KPM buffer for fibers) at room temperature for 10 min in the culture chamber. Fixed samples were then blocked with 0.4% $NaBH_4$ three times, 10 min each, and permeabilized with 0.5% Triton X-100 in PBS (PBS) when necessary. After blocking with 2% BSA (BSA) at room temperature for 6 h, samples were incubated with 1CB4 or anti-As_SRP-1 or anti-pY (Millipore) antibodies (1:200 in PBS with 2% BSA) overnight at 4 °C. After washing three times with PBS, samples were stained with Alexa Fluor 488-conjugated goat anti-mouse or Rhodamine-conjugated goat anti-rabbit secondary antibodies (Molecular Probes; 1:400 in PBS with 2% BSA) at room temperature for 1 h. Images were obtained with a confocal laser scanning microscope (Olympus).

MO Fusion Assay. Spermatids were isolated and incubated in HKB buffer no SAS, SAS, PMSF-treated SAS, or PHE-treated SAS for 10 min at 37 °C as described above. The treated cells were stained with FM1-43FX (Molecular Probes) at 5 μg/mL for 1 min to visualize the PM and MO upon fusion (5). Images were captured using a confocal laser scanning microscope (Leica)

Cryo-Immuno-EM. Ultrathin cryomicrotomy and immunogold labeling were performed according to the protocol of Tokuyasu (6, 7). Sperm were fixed for 30 min with 4% paraformaldehyde in HKB buffer containing 0.1% glutaraldehyde, and infused with 2.3 M sucrose overnight at room temperature for cryoprotection. Fixed sperm were then mounted and snap-frozen in liquid nitrogen on cryospecimen pins for cross-sectioning. Ultrathin cryosections (70–90 nm thick) were cut and transferred to 200-mesh grids with a Leica ultramicrotome. Grids containing these cryosections were then blocked with 5% goat serum for 30 min, followed by incubation with 1CB4 antibody (1:20) for 1 h. After rinsing four times for 5 min each, the grids were transferred to droplets of 15 nm immunogold-conjugated goat anti-mouse secondary antibody (Sigma; 1:20). The diluent for immuno-gold incubations and intermediate rinses was PBS containing 0.15% glycine and 1% BSA. Finally, antibody-labeled grids were fixed with 2.5% glutaraldehyde in PBS for 5 min, stained with 4% uranyl acetate after rinsing with water, transferred to 1% methylcellulose droplets and picked up using a platinum wire loop. Excess embedding solution was removed using filter paper and the grids were allowed to air dry. Images were captured on an FEI 20 transmission electron microscope (FEI).

1. Abbas M, Cain GD (1979) In vitro activation and behavior of the ameboid sperm of Ascaris suum (Nematoda). *Cell Tissue Res* 200:273–284.
2. Italiano JE, Jr., Roberts TM, Stewart M, Fontana CA (1996) Reconstitution in vitro of the motile apparatus from the amoeboid sperm of Ascaris shows that filament assembly and bundling move membranes. *Cell* 84:105–114.
3. Buttery SM, Ekman GC, Seavy M, Stewart M, Roberts TM (2003) Dissection of the Ascaris sperm motility machinery identifies key proteins involved in major sperm protein-based amoeboid locomotion. *Mol Biol Cell* 14:5082–5088.
4. Chi H, et al. (2010) pNovo: de novo peptide sequencing and identification using HCD spectra. *J Proteome Res* 9:2713–2724.
5. Washington NL, Ward S (2006) FER-1 regulates Ca2+ -mediated membrane fusion during C. elegans spermatogenesis. *J Cell Sci* 119:2552–2562.
6. Tokuyasu KT (1986) Application of cryoultramicrotomy to immunocytochemistry. *J Microsc* 143:139–149.
7. Tokuyasu KT (1989) Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. *Histochem J* 21:163–171.

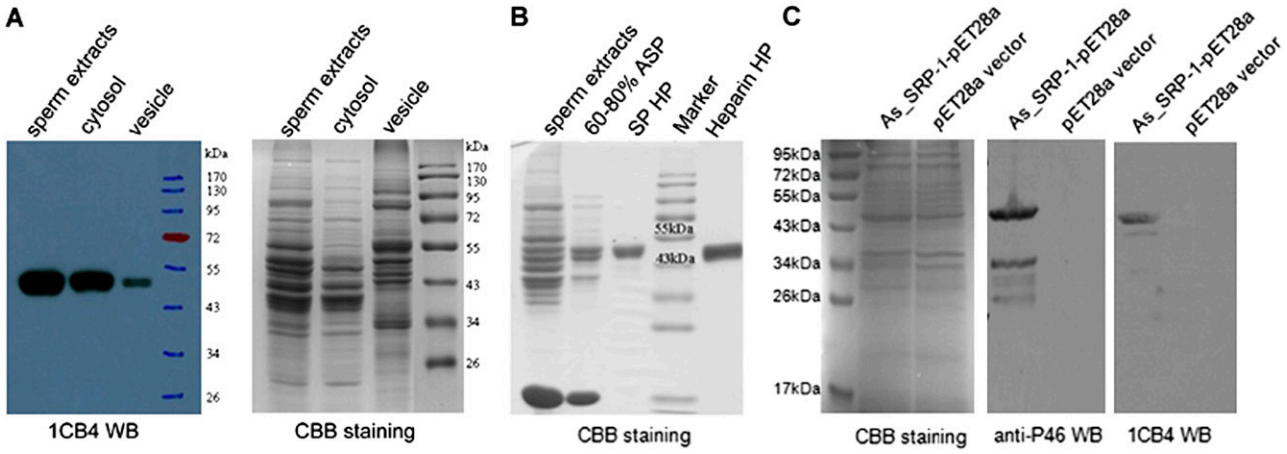

Fig. S1. Identification and confirmation of the 1CB4-recognizing protein as Serpin (As_SRP-1). (*A*) Single band recognized by 1CB4 was detected in sperm extracts, more specifically, in the cytosol and vesicle with SDS/PAGE followed by Western blot. (*B*) Scheme used to purify the *Ascaris* sperm protein recognized by 1CB4. ASP, ammonium sulfate precipitation; Heparin HP, heparin HP affinity chromatography; SH HP, cation exchange columns. (*C*) As_SRP-1 expressed in *E. coli* was detected by SDS/PAGE and Western blots with anti-As_SRP-1 polyclonal antibody (anti-P46) and monoclonal antibody 1CB4.

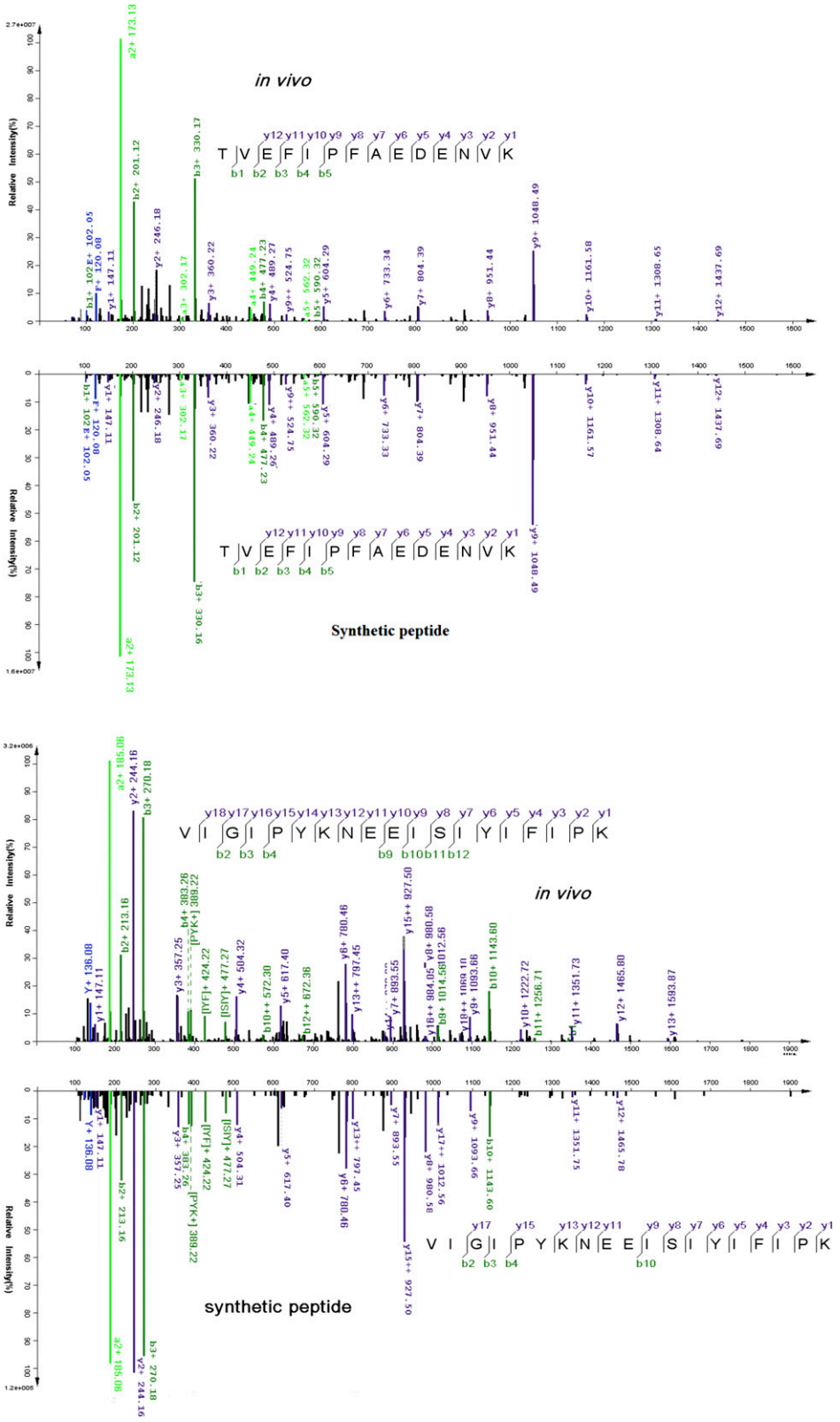

Fig. S2. Verification by synthetic peptides of the pNovo identification of two sequences originated from As_SRP-1. The peptide sequences were interpreted directly from the higher-energy collisional dissociation (HCD) spectra using the pNovo program. Peptides were synthesized for two of them. The original HCD spectra are labeled "in vivo." Displayed below each of them is an HCD spectrum of the synthetic peptide, in perfect agreement with the original spectrum.

As_SRP-1:

Peptide	Spectrum	Modification	pFind-e-value	Mascot-score	pNovo-result	pNovo-score
AFSMTAANFSGICAR	Ascaris25-28_HCD-2.1795.1795.2.dta	4,M(Oxidation);13,C(Carbamidomethyl);	1.87E-28	57.40	MSAFTAANFSGICAR	0.507
FVEIMQEKFEGEIYTMDSDKPSETAK	Ascaris-25-28-hcd-cid-etd2-top1only_090427.2442.2442.3.dta	/	1.77E-47	77.05	VFEIMQEKFEGEIYTMDSDKPSETAK	0.625
AIVNGCDDNTIINYYSDIMHISQQPTTYTLNLANR	Ascaris-25-28-hcd-cid-etd2-top1only_090427.3187.3187.3.dta	4,N(Deamidation);6,C(Carbamidomethyl);13,N(Deamidation);	1.98E-72	75.46	AIVDGCDDNTIINYYSDIMHISQQPTTYTINIANR	0.562
KFVEIMQEK	Ascaris25-28_HCD-2.1572.1572.2.dta	/	6.66E-23	51.48	KFVEIMQEK	0.783
QFIANHPFLFALIKNETILFIGHFLK	Ascaris-25-28-hcd-cid-etd2-top1only_090427.3914.3914.4.dta	15,N(Deamidation);	8.61E-50	47.63	/	/
FEGEIYTMDSDKPSETAKK	Ascaris-25-28-hcd-cid-etd2-top1only_090427.1562.1562.3.dta	/	9.59E-24	32.58	FEGEIYKTMDSDKQVEEK	0.619
EKFFPEENQR	Ascaris25-28_HCD-2.1524.1524.2.dta	/	2.29E-16	38.10	EKFFPEENQR	0.650
RAFSMTAANFSGICAR	Ascaris25-28_HCD-2.1860.1860.2.dta	14,C(Carbamidomethyl);	1.47E-17	58.25	/	/
IMVDMMR	mito_sun_90k_20100902.2433.2433.2.dta	/	4.49E-16	31.97	IMVDMMR	0.782
FGLHEIEHTLNGSK	Ascaris25-28_HCD-2.1866.1866.2.dta	11,N(Deamidation);	8.51E-32	49.47	FGIHEIEHTIDGSK	0.592
TVEFLPFAEDENVK	mito_sun_90k_20100902.3024.3024.2.dta	/	4.69E-21	33.26	TVEFPQTVMDENVK	0.672
FEGEIYTMDSDKPSETAK	Ascaris-25-28-hcd-cid-etd2-top1only_090427.1889.1889.3.dta	/	5.25E-38	74.29	FRCIYTMDSDKPSQTIS	0.351
INQWLR	Ascaris25-28_HCD-2.1650.1650.2.dta	/	2.19E-11	43.95	INQWIR	0.662
DKLIDEQNPDLS	miao-sun-aspn-20101106.2583.2583.2.dta	7,Q(Deamidation);	/	47.83	/	/
NEEISLYIFLPK	mito_sun_90k_20100902.3393.3393.2.dta	/	1.64E-21	30.87	NRICSIMKFPIK	0.622
FFPEENQR	Ascaris25-28_HCD-2.1571.1571.2.dta	/	5.45E-14	46.42	FFPEENQR	0.641
VIGLPYKNEEISLYIFLPK	Ascaris-25-28-hcd-cid-etd2-top1only_090427.3047.3047.3.dta	/	1.57E-33	51.02	VIGIPYKNEEISIYIFIPK	0.585
AFSMTAANFSGICARPTHIR	Ascaris-25-28-hcd-cid-etd2-top1only_090427.2072.2072.3.dta	13,C(Carbamidomethyl);	1.05E-15	40.45	AFSMTAANFSGRRNIFMMR	0.531
ALIEVNEGGTQAAAVSAIEMVFK	Ascaris-25-28-hcd-cid-etd2-top1only_090427.3442.3442.3.dta	/	1.38E-40	80.62	IAIEVNEGSAQAAAVSAIEMVFK	0.566
KINQWLR	Ascaris-25-28-hcd-cid-etd2-top1only_090427.1537.1537.2.dta	3,N(Deamidation);	3.29E-20	28.36	KIDQWIR	0.738
FVEIMQEK	Ascaris-25-28-hcd-cid-etd2-top1only_090427.1379.1379.2.dta	5,M(Oxidation);	5.60E-15	24.71	VFEIMQEK	0.325
NETILFIGHFLK	Ascaris-25-28-hcd-cid-etd2-top1only_090427.2719.2719.2.dta	/	1.25E-29	30.77	/	/
QFIANHPFLFALIK	Ascaris25-28_HCD-2.2534.2534.2.dta	0,Q(Gln->pyro-Glu);	1.19E-29	54.17	MIIFAHNAGPFAIIK	0.576
VIGLPYK	mito_sun_90k_20100902.2372.2372.2.dta	/	2.07E-07	16.26	VIGIPYK	0.656
DMMRTVEFLPFAE	miao-sun-aspN-20101106.3686.3686.2.dta	2,M(Oxidation);3,M(Oxidation);	1.25E-22	27.94	/	/
TVEFLPFAEDENVKVIGLPYK	Ascaris25-28_HCD-2.2274.2274.2.dta	12,N(Deamidation);	4.20E-09	/	/	/
EKFFPEENQRIMV	miao-sun-aspN-20101106.2987.2987.2.dta	/	9.38E-18	/	/	/
SNRPIKR	Ascaris25-28_HCD-2.368.368.2.dta	/	2.67E-09	/	/	/

Fig. S3. Comparison of the de novo sequencing result and the retrospective database search result of As_SRP-1. After the identification and cloning of As_SRP-1, the protein sequence of As_SRP-1 was derived from its full-length cDNA and added to a database. The original MS data were searched against this database using Mascot and pFind, which reidentified As_SRP-1 as the top candidate. Most of the As_SRP-1 peptides identified by database search had been identified by pNovo. Highlighted in yellow are the identical regions between a pNovo-identified sequence and that by database search from the same spectrum.

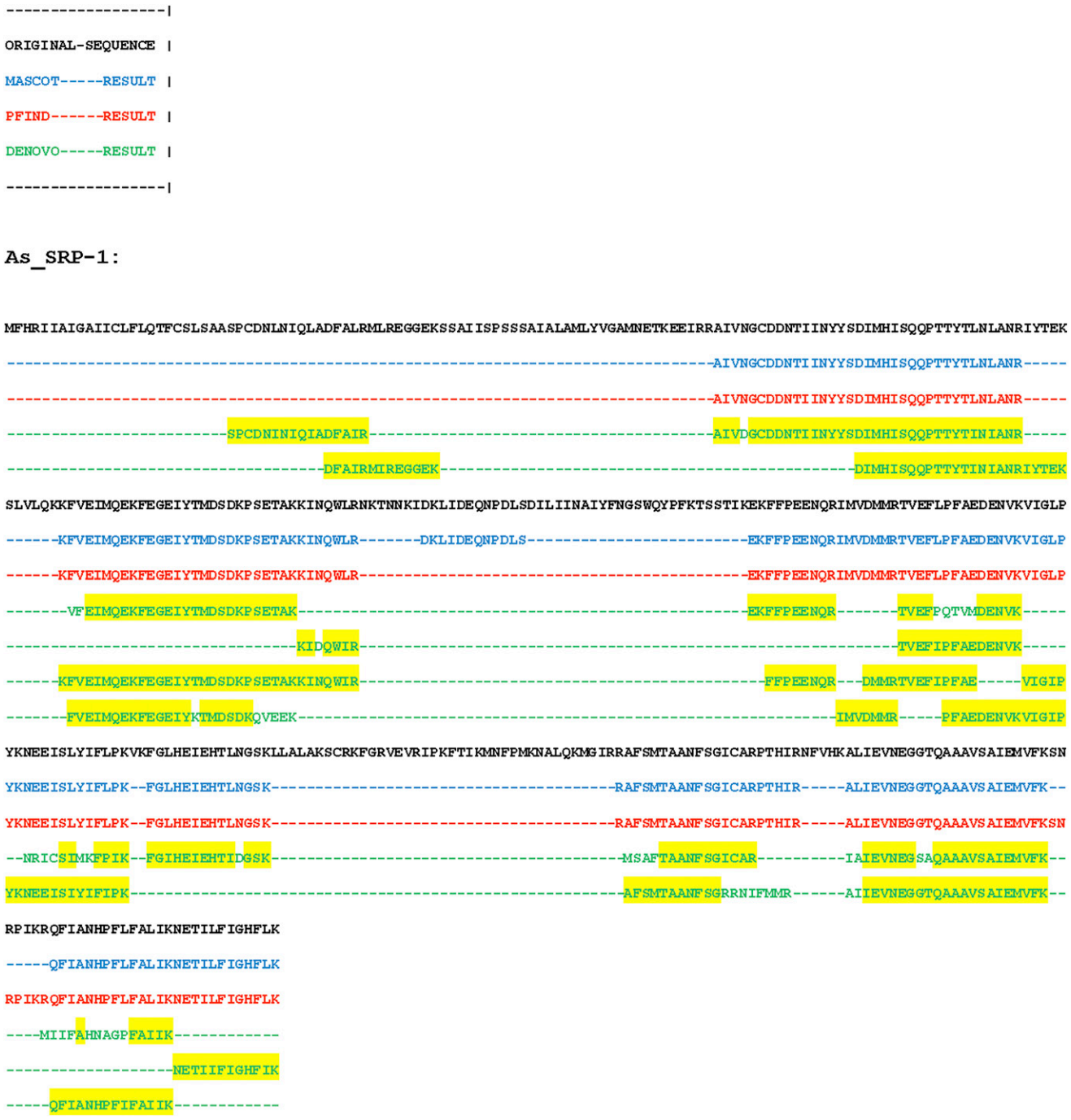

Fig. S4. Sequence coverage of As_SRP-1 by de novo sequencing and database search. Peptide sequences identified by pNovo (green), Mascot (blue), or pFind (red) are mapped to the As_SRP-1 protein sequence. Yellow highlighting indicates the pNovo-identified sequence tags that match perfectly to the As_SRP-1 protein sequence.

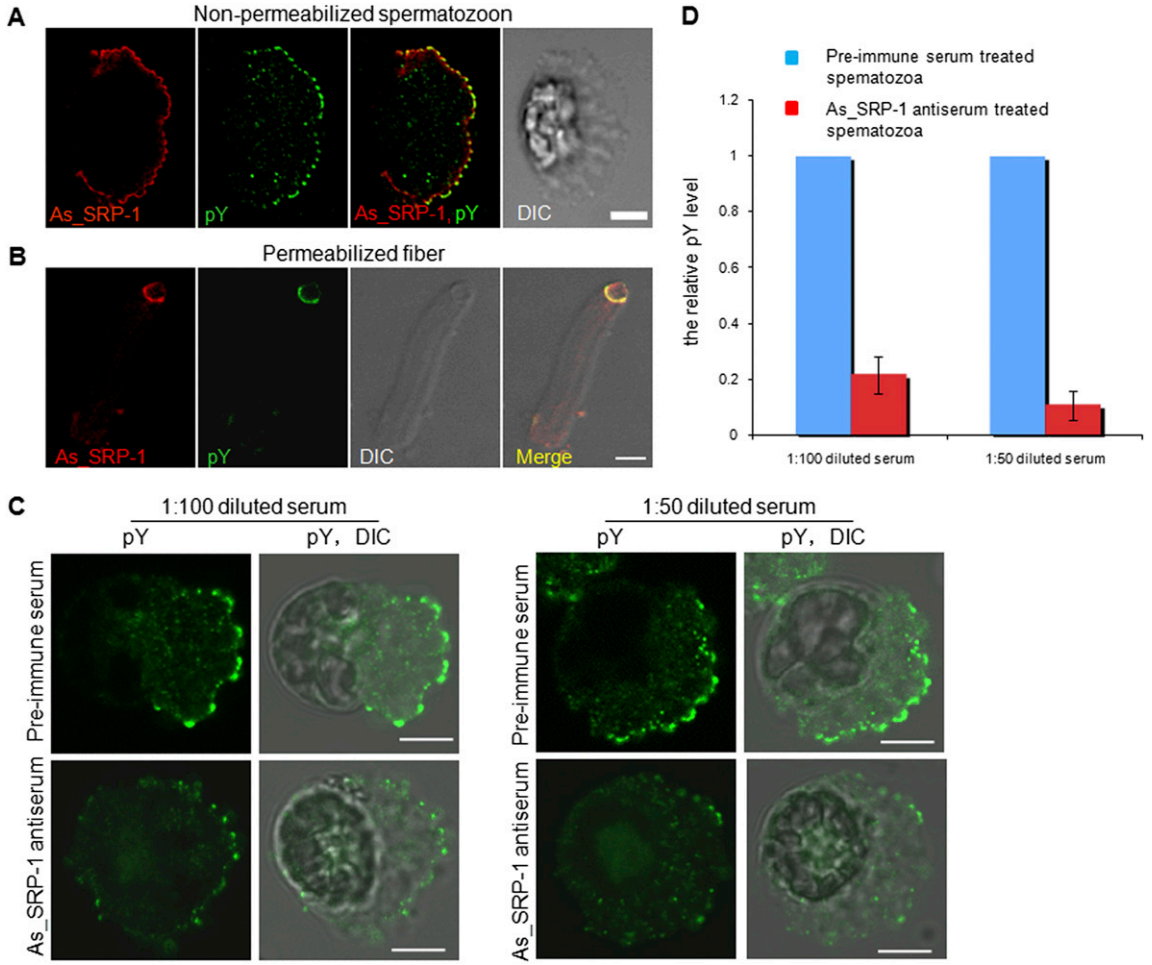

Fig. S5. As_SRP-1 probably regulates *Ascaris* sperm motility through protein tyrosine phosphorylation (pY). (*A*) Colocalization of As_SRP-1 (red) and pY (green) in nonpermeabilized spermatozoa. (Scale bars, 5 μm.) (*B*) Colocalization of As_SRP-1 (red) and pY (green) in permeabilized fibers assembled in vitro. (Scale bars, 5 μm.) (*C*) As_SRP-1 antiserum treatments (1:100 and 1:50) of spermatozoa for 10 min resulted in a decrease of pY signal (green) (confocal imaging) before spermatozoa showing cytoskeletal disassembly and roundup (DIC imaging). Preimmune treatments, control. Spermatozoa were not permeabilized for imaging. (Scale bars, 10 μm.) The relative fluorescence intensity of pY signal at the leading edge of spermatozoa was quantified in (*D*). All data are means ± SD (n = 3 experiments).

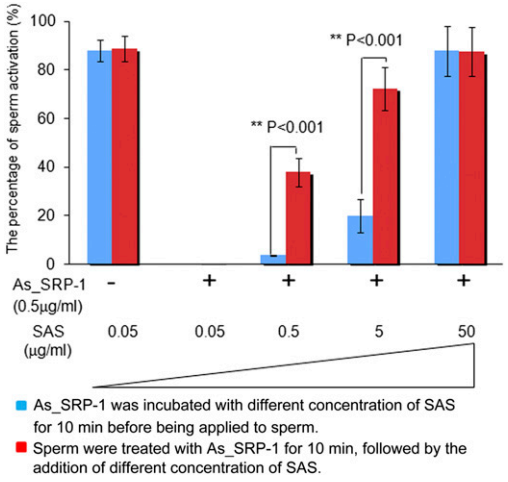

Fig. S6. The inhibitory target of As_SRP-1 is present in SAS. When sperm were treated with the mixture of As_SRP-1 and 0.5 or 5 μg/mL of SAS (blue bars), the sperm activation rate was lower than that from sperm treated with As_SRP-1 first, then followed by SAS addition (red bars). Data are means ± SD (n = 5 experiments). $^{**}P < 0.001$ (Student t test). No difference in sperm activation rate between blue and red bars at 0.05 and 50 μg/mL of SAS was probably because either the SAS concentration was too low (0.05 μg/mL), thus, its sperm activating activity was blocked by 0.5 μg/mL As_SRP-1, or the overwhelming concentration (50 μg/mL) of SAS abolished the inhibitory effect of As_SRP-1.

A

Inhibitors specific for	Inhibitory effect	rescue effect with fresh SAS
Serine peptidases		
PMSF	Yes	Yes
TLCK	No	
TPCK	No	
DCI	Yes	Yes
Apro	No	
SB-TI	No	
Ben	No	
Cysteine peptidases		
E-64	No	
IAM	No	
N-E	Yes	No
Metallopeptidases		
EDTA	No	
PHE	Yes	Yes
Aspartate peptidases		
PePA	No	

B

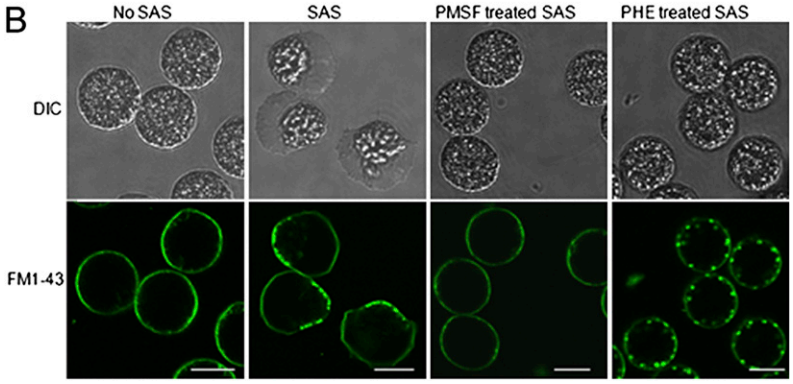

Fig. S7. Serine protease activity in SAS is necessary for sperm activation. (*A*) Summary of inhibitory effects of various protease inhibitors tested on SAS activity of sperm activation. The SAS (0.25mg/mL) was preincubated with various protease inhibitors [1 mM phenylmethanesulfonylfluoride (PMSF), 10 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 2.6 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM 3,4-dichloroisocoumarin (DCI), 0.2 mg/mL aprotinin (Apro), 1 mg/mL Soybean trypsin inhibitor (SB-TI), 10 mM benzamidine (Ben) 0.15 mM E-64, 5 mM iodoacetamide (IAM) 25 mM *N*-ethylmaleimide (*N*-E), 2.5 mM EDTA, 5 mM *o*-phenanthroline (PHE), or 0.5 mM pepstatin A (PePA)], respectively, for 30 min on ice. The spermatids were incubated with treated SAS and checked by light microscopy. The results showed that serine protease, cysteine protease, and metalloprotease, but not aspartate protease activities in SAS might be important for sperm activation. For those inhibitors with inhibitory effect on sperm activation, the treated SAS was replaced by perfusion with fresh SAS to examine the rescue effects. The serine protease inhibitors (PMSF and DCI) and the metalloprotease inhibitor (PHE) inhibited sperm activation through their effect on SAS. The cysteine protease inhibitor *N*-E inhibited sperm directly and irreversibly (it could not be rescued by fresh SAS). (*B*) Examination of MO fusion. Spermatids were incubated with HKB buffer (no SAS), SAS, PMSF-treated SAS or PHE-treated SAS for 10 min. Lipophilic dye FM1-43FX that stains the outer leaflet of the PM was added to visualize the PM and MO upon fusion. *Upper*, DIC images; *Lower*, FM1-43FX staining. Spermatids (no SAS or PMSF-treated SAS) only displayed PM staining and spermatozoa (SAS) showed PM and many bright puncta (fused MOs) around the cell body periphery. Bright puncta were observed in the sperm incubated with PHE-treated SAS although pseudopod formation was inhibited. This assay further eliminated the metalloprotease activity in SAS as being responsible for sperm activation because PHE-treated SAS did induce spermatids to initiate MO fusion with the PM, although pseudopod formation was aborted. (Scale bar, 10 μm.)

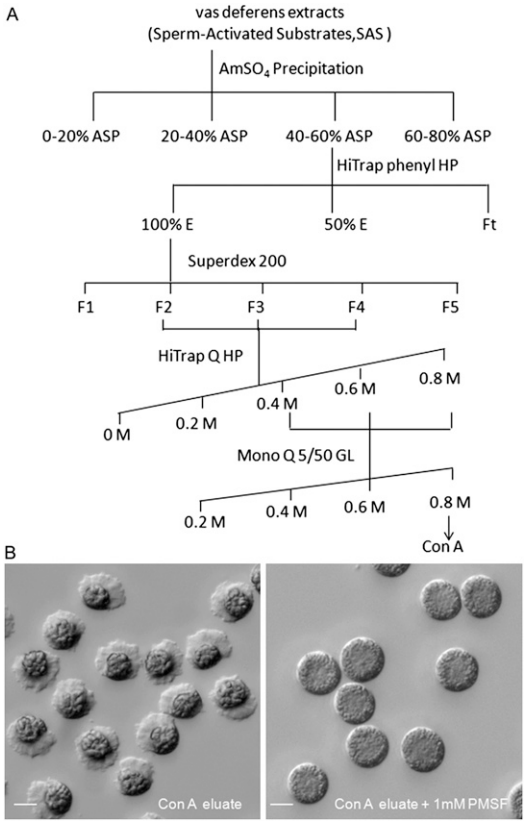

Fig. S8. Enrichment of the sperm activator, a serine protease(s) from SAS. (*A*) Flowchart of enrichment of sperm activator. (*B*) The Con A eluate showed strong sperm activating activity (left) and this activity was inhibited by a serine protease inhibitor PMSF (right). (Scale bar, 10 μm.)

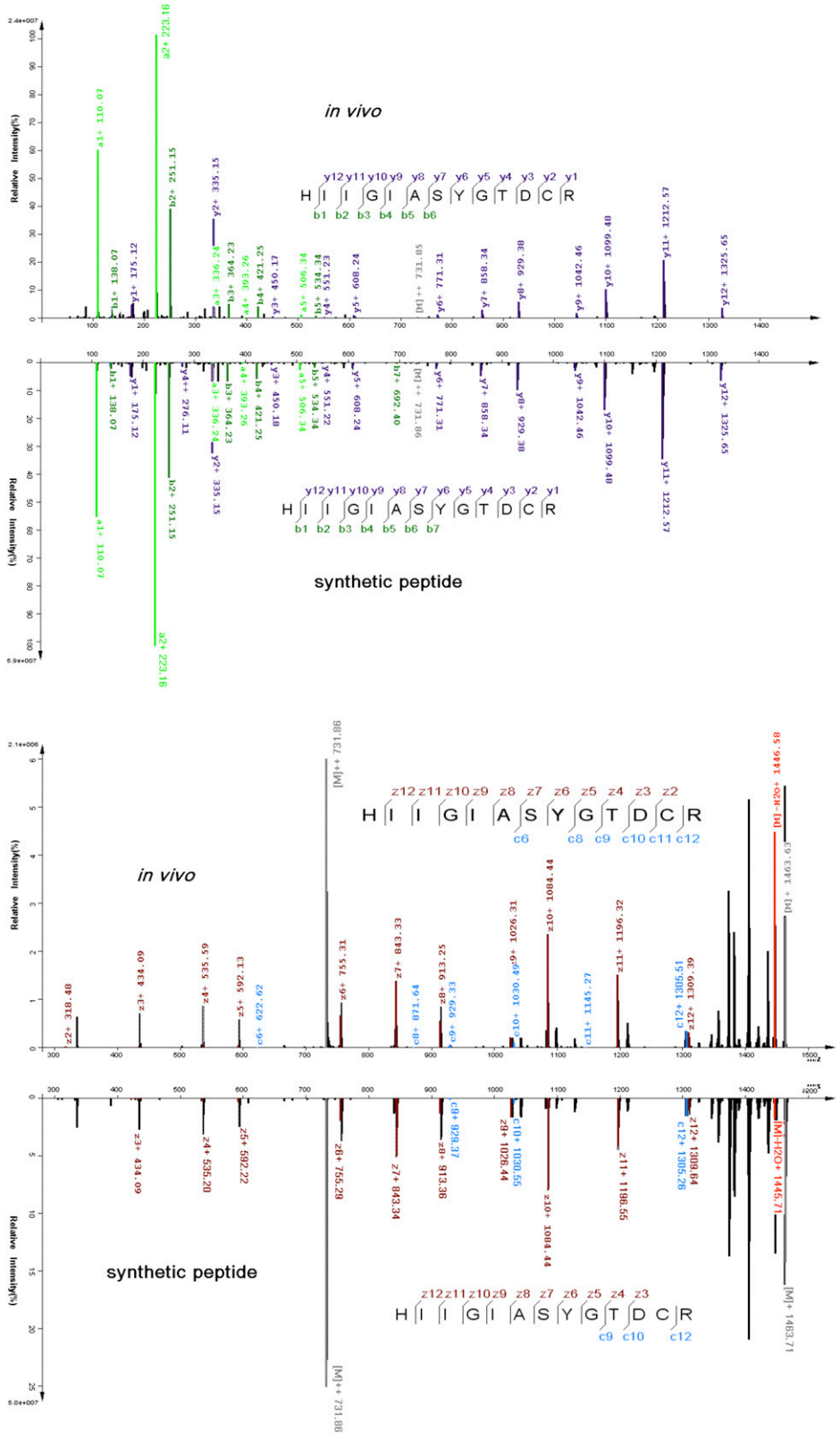

Fig. S9. Fragmentation spectra of a synthetic peptide validating the pNovo identification of a sequence originated from As_TRY-5. The HCD (*Upper*) and ETD (*Lower*) spectra of a synthetic peptide, which was synthesized according to a pNovo sequence identification, are fully consistent with the interpretation of the original spectra (labeled in vivo) by pNovo.

As_TRY-5:

Peptide	Spectrum	Modification	pFind-e-value	Mascot-score	pNovo-result	pNovo-score
DIRPFNNFICKYTGVCDDE	miao-sun-aspN-20101106.3369.3369.2.dta	6,N(Deamidation);	/	32.36	/	/
DISDAIILQRN	miao-sun-aspN-20101106.2971.2971.2.dta	/	1.88E-12	34.75	/	/
TIEDISDAIILQR	mito_sun_90k_20100902.4204.4204.2.dta	/	6.66E-46	56.13	ITEDISDAIIIQR	0.749
TIQMALDYRDEFDEWEK	mito_sun_90k_20100902.3054.3054.3.dta	4,M(Oxidation);	2.57E-24	11.72	TIQMTDFEDRYDIAQSEK	0.731
KHIIGIASYGTDCR	mito_sun_90k_20100902.2141.2141.2.dta	13,C(Carbamidomethyl);	1.13E-13	22.67	KHCHYEMIGIIVF	0.530
DEWEKLSELAGGIFT	miao-sun-aspN-20101106.4552.4552.2.dta	/	/	70.44	EDWEKISEIANIFT	0.737
DAIILQRN	miao-sun-aspN-20101106.2462.2462.2.dta	/	9.77E-13	15.56	GEIIIQRN	0.776
TQLASWGSDASR	mito_sun_90k_20100902.2235.2235.2.dta	/	1.64E-20	63.76	QTIASWGSDASR	0.775
IFPAFLAQPLCLASK	mito_sun_90k_20100902.3302.3302.2.dta	11,C(Carbamidomethyl);	8.87E-19	58.10	MKPAFIAQPICIASK	0.806
HIIGIASYGTDCR	mito_sun_90k_20100902.2268.2268.2.dta	12,C(Carbamidomethyl);	6.09E-26	68.45	HIIGIASYGTDCR	0.816
LSELAGGIFTDIRPFNNFICK	mito_sun_90k_20100902.3335.3335.3.dta	16,N(Deamidation);20,C(Carbamidomethyl);	5.39E-25	37.74	ISEIAGGIFTDIRMINNFICK	0.720
IDLSTIPCPIESTFR	mito_sun_90k_20100902.3082.3082.2.dta	8,C(Carbamidomethyl);	5.12E-21	70.26	EVISTIPCPIESTFR	0.732
DCRTIQMAL	miao-sun-aspN-20101106.2621.2621.2.dta	2,C(Carbamidomethyl);7,M(Oxidation);	1.78E-16	30.69	DCRTIQMAI	0.749
DLSTIPCPIESTFR	miao-sun-aspN-20101106.3320.3320.2.dta	7,C(Carbamidomethyl);	1.49E-16	31.24	SIDTIPCPIESTFR	0.688
DDNKTIEDIS	miao-sun-aspN-20101106.2426.2426.2.dta	/	/	11.20	/	/
DASRMKTITTLTKI	miao-sun-aspN-20101106.2817.2817.3.dta	/	8.35E-36	50.53	/	/
DYRDEF	miao-sun-aspN-20101106.2630.2630.2.dta	/	/	11.59	/	/
DILSEYILDDNKTIE	miao-sun-aspN-20101106.3807.3807.2.dta	/	/	52.08	/	/
DASRMKTITTLTKIDLSTIPCPIESTFR	miao-sun-aspN-20101106.3694.3694.3.dta	21,C(Carbamidomethyl);	2.01E-37	33.88	KTWHGHRTTITKIDISTICRTMKSIPN	0.595
DEFDEWEKLSELAGGIFT	miao-sun-aspN-20101106.5047.5047.2.dta	/	/	61.02	EDFDEWEKISEISNIMV	0.653
GDLAIVELDAK	mito_sun_90k_20100902.2634.2634.2.dta	/	8.16E-16	32.98	GDIAIVEIDAK	0.732
DIRPFNNFICKYTGVCD	miao-sun-aspN-20101106.3441.3441.2.dta	10,C(Carbamidomethyl);16,C(Carbamidomethyl);	1.85E-16	25.76	KCSPFRISICRYNNGVM	0.664
YTGVCDDEDILSEYILDDNK	mito_sun_90k_20100902.3203.3203.2.dta	5,C(Carbamidomethyl);	3.10E-18	41.85	EDDDCRTYIISEYIIDDNK	0.685
DSGAGMVYSNKGRKHIIGIASYGTDCRTIQMAL	miao-sun-aspN-20101106.2852.2852.4.dta	26,C(Carbamidomethyl);31,M(Oxidation);	4.61E-08	/	/	/
DIRPFNNFICKYTGVC	miao-sun-aspN-20101106.3413.3413.2.dta	6,N(Deamidation);10,C(Carbamidomethyl);16,C(Carbamidomethyl);	1.46E-05	/	/	/
DECQRNQIKALTSKIRNVIIPKVFI	miao-sun-aspN-20101106.3864.3864.4.dta	3,C(Carbamidomethyl);	0.00394	/	/	/

Fig. S10. Comparison of the de novo sequencing result and the retrospective database search result of As_TRY-5. After the identification and cloning of As_TRY-5, the original MS data were searched against a database containing this protein using Mascot and pFind, and As_TRY-5 was reidentified as the top candidate. Most of the As_TRY-5 peptides identified by database search had been identified by pNovo. Highlighted in yellow are the identical regions between a pNovo-identified sequence and that by database search from the same spectrum.

Fig. S11. Sequence coverage of As_TRY-5 by de novo sequencing and database search. Peptide sequences identified by pNovo (green), Mascot (blue), or pFind (red) are mapped to the As_TRY-5 protein sequence. Yellow highlighting indicates the pNovo-identified sequence tags that match perfectly to the As_TRY-5 protein sequence.