Isolation of IRS-2 cDNA. Ten different arthropod serpins whose sequences were publicly disclosed at that time were used in multiple alignments to reveal two conserved domains in arthropod serpins. A radioactively labeled DNA probe was prepared from the PCR product, obtained with degenerated primers designed based on the sequences of these two domains (Fwd: 5′-AACGC(C/T)(A/G)TCTACTT(C/T)AA(A/G)GG-3′, Rev: 5′-GGATGAAGAA (G/C)A(G/T)GAA(A/T/C/G)GG-3′) using a DecaLabel DNA kit (Fermentas Life Sciences). The probe was used for the screening of a λ phage cDNA library constructed from the salivary glands of partially fed (d 5 after attachment) *I. ricinus* female adults. The cDNA library was prepared using the SMART™ cDNA library construction kit (ClonTech) according to the manufacturer's protocol. Phages were plated on 150-mm Petri dishes, and the print was taken onto the nylon membrane Hybond™ (Amersham Biosciences). The DNA was linked to the membranes using Stratalinker™ 1800 (Stratagene), the membranes were preincubated (30 min at 63.5°C with 2% salmon sperm in 0.1% SDS, 300 mM NaCl, 30 mM natrium citrate, pH 7), and then 25 μl of the denatured (97°C; 10 min) probe was added to each hybridization tube. Hybridization was done overnight at 63.5°C. After hybridization, the membranes were washed 3 × 30 min with decreasing concentrations of NaCl (450, 150, and 75 mM) in the presence of 0.1% SDS. After washing, the membranes were dried and exposed on Kodak X-Omat™ LS film using a Kodak X-Omat™ cassette at –80°C. Positive single clones were isolated after a second screen, excised, and subcloned into pTriplEx vector according to the manufacturer's protocol and sequenced in an ABI PRISM 3130xl sequencer (Applied Biosystems).

Bioinformatics tools. BLASTx algorithm on the server http://blast.ncbi.nlm.nih.gov/Blast.cgi [1] was used for similarity searches of IRS-2 with other members of the serpin superfamily. The presence of signal sequences on the proteins was determined using SignalP 3.0 server http://www.cbs.dtu.dk/services/SignalP/, [2] and their mol wt and pI were determined using ProtParam program on ExPASy proteomic server http://www.expasy.org/tools/protparam.html.[3]

cDNA preparation and quantitative PCR. Ticks used for tissue isolation were fed on guinea pigs complying with Act No. 207/2004 Coll. and approval AVCR 51/2005 given by the respective committee of Czech Academy of Sciences. Tissues used for RNA isolation were dissected from five feeding stages of adult females: for unfed ticks, 20 females were used; for next stages, 10–15 females were dissected. RNA was isolated with a NucleoSpin RNA XS kit (Macherey-Nagel). The amount of total RNA used for cDNA preparation was set to 0.3 μg for all samples. cDNA was prepared using an Avian Enhanced First-strand Synthesis Kit and diluted 20 times in deionized water to achieve optimal amplification conditions (linear amplification between 20th and 30th cycle). The program for quantitative PCR was: 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. All samples were prepared in triplicates. Dual-labeled probe from Universal probe library (Roche) and IRS-2-specific primers were used for quantitative RT-PCR. Data were obtained with Rotor-Gene RG3000 (Corbett Research) and analyzed using the Pfaffl equation. [4] Based on our previous experiences with various housekeeping genes (data not shown), we decided on the tick ferritin (NCBI GenBank Acc. No. AF068224) [5] for use as a reference gene.

Preparation of recombinant IRS-2. Primers for amplifying IRS-2 cDNA for overexpression in bacteria were designed to amplify the gene without its signal peptide sequence and to introduce restriction sites for *Nde*I and *Xho*I on its 5′ and 3′ ends, respectively, for subcloning purposes. To improve gene expression levels in bacteria, codons for rare tRNAs in *Escherichia coli* were replaced by introducing synonymous mutations into the forward

primer. (Fwd: 5′-GCCATATGCAAGAAGAAGCCAAGCTCACCAAGGCCAACAACCG T-3′, Rev: 5′-GCCTCGAGTTATCACAGCTTGTTAACCTGTCCCACGAAAAAAATGT CATC-3′). The gene was PCR amplified using cDNA isolated from adult female tick salivary glands 5 d after attachment to guinea pigs as a template (see below). The amplified product was cloned into Zero Blunt™ TOPO PCR vector (Invitrogen) and sequenced to verify the integrity of the frame. Then the IRS-2 gene was subcloned into the expression vector pET 17b (Novagen) using *Nde*I and *Xho*I restriction enzymes (Invitrogen). Gene overexpression was done in BL21(DE3)pLysS cells (Invitrogen) grown in ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) selection and isopropyl β-D-1-thiogalactopyranoside (IPTG) was used for gene expression induction (0.5 mM). IPTG was added when the OD_{600} of the culture reached approximately 0.7 and bacterial growth in the presence of IPTG continued for 3.5 h. Subsequently, the cells were harvested by centrifugation (10,000 × g, 5 min), and the resulting pellet was resuspended in 20 mM Tris-HCl, pH 8. The cells were disrupted by sonication (medium amplitude, 3 × 1 min), and centrifuged (10,000 × g; 10 min), resulting in a pellet that, among other insoluble material, contained the rIRS-2 in inclusion bodies. The pellet was resuspended in inclusion bodies isolation buffer (20 mM Tris-HCl, 1% Triton X-100; pH 8) and sonicated. Next, the suspension was centrifuged (10,000 × g; 10 min), and the resulting pellet was washed four times with 20 mM Tris-HCl, pH 8, resulting in a final pellet that contained mainly inclusion bodies of nonsoluble, overexpressed recombinant IRS-2. Inclusion bodies were dissolved by stirring in 6 M guanidine hydrochloride, pH 8, with 10 mM dithiotreitol for 1 h at room temperature. The solution was centrifuged at 10,000 × g for 15 min, the resulting pellet was discarded, and the resulting supernatant containing the denatured protein content of the inclusion bodies was dissolved into 150-fold volume of refolding buffer (20 mM Tris-HCl, 0.25 M L-arginine; pH 8). The buffer remained overnight at 4°C, and the precipitated protein was removed by filtration through filtration paper and Steritop-GP (Millipore). The protein solution was then

concentrated using a stirred chamber concentrator (Millipore) down to the volume of 10 ml. The concentrated protein was dialyzed against 20 mM Tris-HCl, pH 8. Refolded and concentrated IRS-2 was purified on a Mono-Q column with 0–1 M gradient of NaCl using ÄKTA FPLC system (Pharmacia) with purity > 99% according to SDS-PAGE. During Mono Q purification step, a major symmetric peak, corresponding to properly folded IRS-2 (as verified on SDS-PAGE and by comparing inhibitory activity of isolated fractions) was separated from other contaminants and potentially misfolded protein. Endotoxin was removed by phase separation using Triton X-114 (Sigma-Aldrich), and traces of detergent were removed using SM2 BioBeads (BioRad) as described elsewhere [6]. Endotoxin level was estimated using Limulus amobecyte lysate QCL-1000® (Lonza), following the manufacturer's instructions. Endotoxin contamination did not exceed 0.1 EU/ml in any of the protein samples used for immunological assays, and the protein samples did not induce production of nitric oxide by Dendritic Cells (data not shown), further verifying the LPS decontamination in the IRS-2 preparation.

Serine protease inhibition assays. All assays were performed at 30^{o}C in triplicates. For the initial screen, 400 nM of protein was pre-incubated with each enzyme for 10 min before the addition of the corresponding substrate; *t*-test was used for statistical analysis of the observed inhibition in the presence of 400 nM IRS-2, and statistical significance was considered when $p < 0.05$ when comparing the enzymatic activity in the presence or absence of the inhibitor. For IC_{50} estimation of IRS-2, increasing concentrations of the inhibitor were pre-incubated for 10 min with the enzymes targeted by IRS-2, and the reaction was started by adding the corresponding substrates. All experiments were performed in triplicate (for each enzyme and each concentration of IRS-2). The mean percentage of remaining enzymatic activity in the presence of various IRS-2 concentrations compared with the control enzymatic activity (in the absence of IRS-2) was plotted against the concentration of IRS-2 used in the assays and in logarithmic scale. Finally, sigmoidal fit of the data gave the estimate of the IC_{50} of IRS-2 for

the various enzymes. To determine whether IRS-2 is a fast- or slow-binding inhibitor for the targeted enzymes, either the enzyme was pre-incubated with the inhibitor for 10 min and the addition of substrate followed, or the substrate was pre-incubated with the inhibitor for 10 min and the addition of the enzyme followed. In both cases, the hydrolysis rate of the fluorescent substrate was estimated from the slope that results from the linear fit (arbitrary fluorescence units per sec; $r^2 > 0.95$) of the data (each experiment was performed in triplicate, and the mean of the three experiments was plotted).

All enzymes used were of human origin, purified or recombinant. Thrombin, α-chymotrypsin, plasmin, and chymase were purchased from Sigma; β-tryptase was purchased from Promega; factor Xa was purchased from EMD Biosciences; factor XIIa was purchased from Haematologic Technologies Inc.; kallikrein was purchased from Fitzgerald Industries International; elastase was purchased from Elastin Products; cathepsin G, Factor XIa, uPA, and tPA were from Molecular Innovations; matriptase was from R&D Systems; proteinase 3 was from Merck; and sequencing-grade trypsin was purchased from Roche. The amount of targeted enzyme used in each assay is shown in Table I. Assay buffers were: for elastase, proteinase 3 and chymase, 50 mM Hepes buffer, pH 7.4, 100 mM NaCl, 0.01% Triton X-100; for trypsin and α-chymotrypsin, factor XIa, factor XIIa, and thrombin, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 20 mM $CaCl_2$, 0.01% Triton X-100; for β-tryptase, 50 mM Tris-HCl, pH 8, 50 mM NaCl, 0.05% Triton X-100; for kallikrein, matriptase, and plasmin, 20 mM Tris–HCl, pH 8.5, 150 mM NaCl, 0.02% Triton X-100; for factor Xa, 20 mM Tris-HCl, pH 8, 200 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA; for uPA and tPA, 20 mM Tris-HCl, pH 8.5, 0.05% Triton X-100; and for cathepsin G, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Triton X-100. The substrates used were Suc-Ala-Ala-Pro-Val-AMC for elastase and proteinase 3, Boc-Asp-Pro-Arg-AMC for thrombin and plasmin, Boc-Gln-Ala-Arg-AMC for trypsin, factor XIa and uPA (Sigma), Boc-Phe-Ser-Arg-AMC for β-tryptase, Suc-Leu-Leu-Val-Tyr-AMC for chymase

(Bachem Bioscience, Inc.), Suc-Ala-Ala-Pro-Val-AMC for α-chymotrypsin and chymase (EMD Biosciences), and methylsulfonyl-D-cyclohexylalanyl-Gly-Arg-AMC acetate for factor Xa, factor XIIa, t-PA, matriptase, and kallikrein (American Diagnostica Inc.). All substrates were used in 250 μM final concentration in all assays. Substrate hydrolysis rate was followed in a Spectramax Gemini XPS 96-well plate fluorescence reader (Molecular Devices) using 365 nm excitation and 450 nm emission wavelength with a cutoff at 435 nm.

MPO activity. MPO was performed as an index of granulocyte recruitment into the hind paw inflamed tissue according to a modified protocol of Bradley et al. [7]. Briefly, the injected paws were cut at 4 h post-injection (the time point at which edema peaks) and weighed prior to their homogenization in 2 ml of 0.5% hexadecyl-trimethylammonium bromide phosphate-buffered solution (pH 6.0). The homogenates were then centrifuged at 13,000×g for 3 min, and the supernatants were collected and frozen until use. Three aliquots of each supernatant were then transferred into 96-well plates before the addition of a solution containing o-dianisidine (0.2 mg/ml) and H_2O_2 (0.0006%). In parallel, dilutions of pure myeloperoxidase (Sigma) were used for the construction of a standard curve (OD as a function of units of enzyme activity). OD readings at 450 nm were taken at 1 min (time point corresponding to the linear portion of the enzymatic reaction) using a spectrofluorometer linked to SOFTmax Pro 3.0 software (Molecular Devices). MPO activity detected in the paws was expressed as units of enzyme/g of tissue. A unit of MPO activity was defined as that converting 1 μm of hydrogen peroxide to water in 1 min at 22°C. Alternatively The MPO activity of samples was compared to a standard curve of neutrophils [8]. Briefly, 10 ml of sample was mixed with 200 ml of 50mM phosphate buffer pH 6.0, containing 0.167mgml^{-1} O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results are presented as the MPO activity (number of neutrophils per mg of tissue).

Analysis of PCMC derived proteases activity. On d 2 after the change of growth medium, PCMCs were washed and resuspended in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.36

mM Na_2HPO_4, 5.55 mM glucose; pH 7.4) to a concentration of 10^6 cells/ml. 10^5 of PCMCs were incubated at 37°C and 5% CO_2 for 40 min with different concentrations of IRS-2 in a total volume of 200 µl. Then the cells were activated with 2 µM of ionomycin in the presence or absence of IRS-2 and incubated for additional 30 min. Untreated cells with ionomycin were used as a negative control, and cells treated with ionomycin only (in the absence of IRS-2) were used as a positive control. For enzymatic assays 20 µl (final concentration 100 µM) of the chromogenic substrate MeO-Suc-Arg-Pro-Tyr-pNA (Chromogenix) was added after 30 min of preincubation with IRS-2, and the substrate hydrolysis rate was measured at 405 nM using Sunrise™ reader (TECAN) with the methodology described above. For western blot analysis, cells were preincubated for 30 min with different concentrations of IRS-2, centrifuged at 1100 rpm for 7 min, and the supernatant transferred into a clean tube. The pellet was resuspended in 20 µl of 1× Laemmli sample buffer (2% SDS, 5% 2-mercaptoehtanol, 10% glycerol, 0.002% bromophenol blue, 60 mM Tris-HCl). Twenty µl of both supernatant and resuspended cells was loaded onto 12% SDS polyacrylamide gel and transferred to nitrocellulose filters. Filters were blocked with 5% BSA in TBS-T (10 mM Tris-HCl, pH 7.7, 0.15 M NaCl, 2 % Tween-20) for 1 h at room temperature. Custom polyclonal rabbit antisera raised against mMCP-4 were diluted 1:500 in TBS-T and incubated overnight at 4°C with the filters. After three washing steps with TBS-T for 3 × 5 min, donkey anti-rabbit Ig conjugated to horseradish peroxidase (Amersham Biosciences) was diluted 1:2000 in TBS-T and incubated for 1 h with the filters. After 4 washing steps with TBS-T for 4 × 5 min, an ECL detection kit (Amersham Biosciences) was used for results visualization.

Results

Characterization of chymase, cathepsin G and thrombin inhibition by IRS-2

IRS-2 inhibited cathepsin G and chymase in equimolar ratio. Furthermore, when lowering the concentrations of cathepsin G or chymase, lower concentrations of IRS-2 were necessary for

50% inhibition of these two enzymes. In fact, there was a linear correlation between the amount of enzyme used in the assays and the observed IC_{50}. Therefore, IRS-2 can be considered as a tight binding inhibitor of these two enzymes (Fig. S1 A, B).

On the contrary, IRS-2 behaves like a classical Michaelis-Menten inhibitor (large excess of inhibitor compared with the amount of the enzyme used in the assays) for thrombin (Table I; Fig. S1 C). Furthermore, inhibition of chymase was higher when the enzyme was preincubated with IRS-2, and a competition between the inhibitor and the substrate was observed when they were added simultaneously to the reaction, suggesting slow binding (Table I; Fig. S2 A). Preincubation of IRS-2 with cathepsin G did not change the observed inhibition rate, indicating fast binding (Table I; Fig. S2 B).

Preincubation of IRS-2 with thrombin improved the observed enzymatic inhibition, indicating that IRS-2 is a slow-binding inhibitor of thrombin with low ability to compete with the artificial substrate used for detection of enzymatic activities (Table I; Fig. S2 C).

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Table S1. Crystallographic dataset and refinement statistics

Data collection statistics	
Space group	$P4_3$
Cell parameters (Å)	84.6, 84.6, 124.4
Number of molecules in AU	2
Wavelength (Å)	0.953
Resolution (Å)	30.0 – 1.80 (1.86 – 1.80)
Number of unique reflections	80,249 (7,790)
Redundancy	3.8 (3.3)
Completeness (%)	99.6 (97.3)
R_{merge}[a]	4.6 (35.8)
Average I/σ(I)	32.8 (2.3)
Wilson B ($Å^2$)	23.2
Refinement statistics	
Resolution range (Å)	30.0 – 1.80 (1.86 – 1.80)
No. of reflections in working set	76,177 (5,415)
No. of reflections in test set	4,026 (285)
R value (%)[b]	17.1 (24.7)
R_{free} value (%)[c]	21.2 (31.5)
RMSD bond length (Å)	0.011
RMSD angle (°)	1.22
Number of atoms in AU	7,003
Number of protein atoms in AU	6,111
Number of water molecules in AU	875
Mean B value protein / solvent ($Å^2$)	23.6/38.6
Ramachandran plot statistics	
Residues in favored regions (%)	91.3
Residues in allowed regions (%)	5.7

Data in parentheses refer to the highest-resolution shell.

$$R_{merge} = \Sigma_{hkl}\Sigma_i I_i^9 - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i\ I_i(hkl),$$

where the $I_i(hkl)$ is an individual intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity of reflection hkl with summation over all data.

$$R\ value = ||F_o| - |F_c||/|F_o|,$$

where F_o and F_c are the observed and calculated structure factors, respectively. R_{free} is equivalent to R value but is calculated for 5% of the reflections chosen at random and omitted from the refinement process.

Table S2. Concentration of used enzymes and remaining enzymatic activity. The values represent the mean remaining enzymatic activity in the presence of IRS-2 with the standard error of the mean. Enzymes in bold were inhibited with a statistical significance (*t*-test; $P < 0.05$).

Enzyme tested	Final concentration of enzyme used [nM]	% remaining enzymatic activity in the presence of 0.4µM IRS-2
kallikrein	0.8	92.5 ± 5.2
tryptase	0.27	106.8 ± 11.2
uPA	0.7	87.6 ± 2.9
factor Xa	0.8	97.8 ± 0.6
granzyme B	20	105.8 ± 2.7
matriptase	1.2	98,6 ± 3
elastase	0.01	101.1 ± 2.1
proteinase 3	10	91.45 ± 1.3
a-chymotrypsin	**0.05**	**0.3 ± 0.2**
chymase	**1.8**	**1 ± 0.5**
factor XIIa	1.2	96,5 ± 1.6
factor XIa	0.06	105.3 ± 6.7
plasmin	0.25	103.2 ± 4.8
thrombin	**0.01**	**11.6 ± 0.9**
trypsin	**0.25**	**55.2 ± 4**
cathepsin G	**10**	**4.9 ± 0.9**

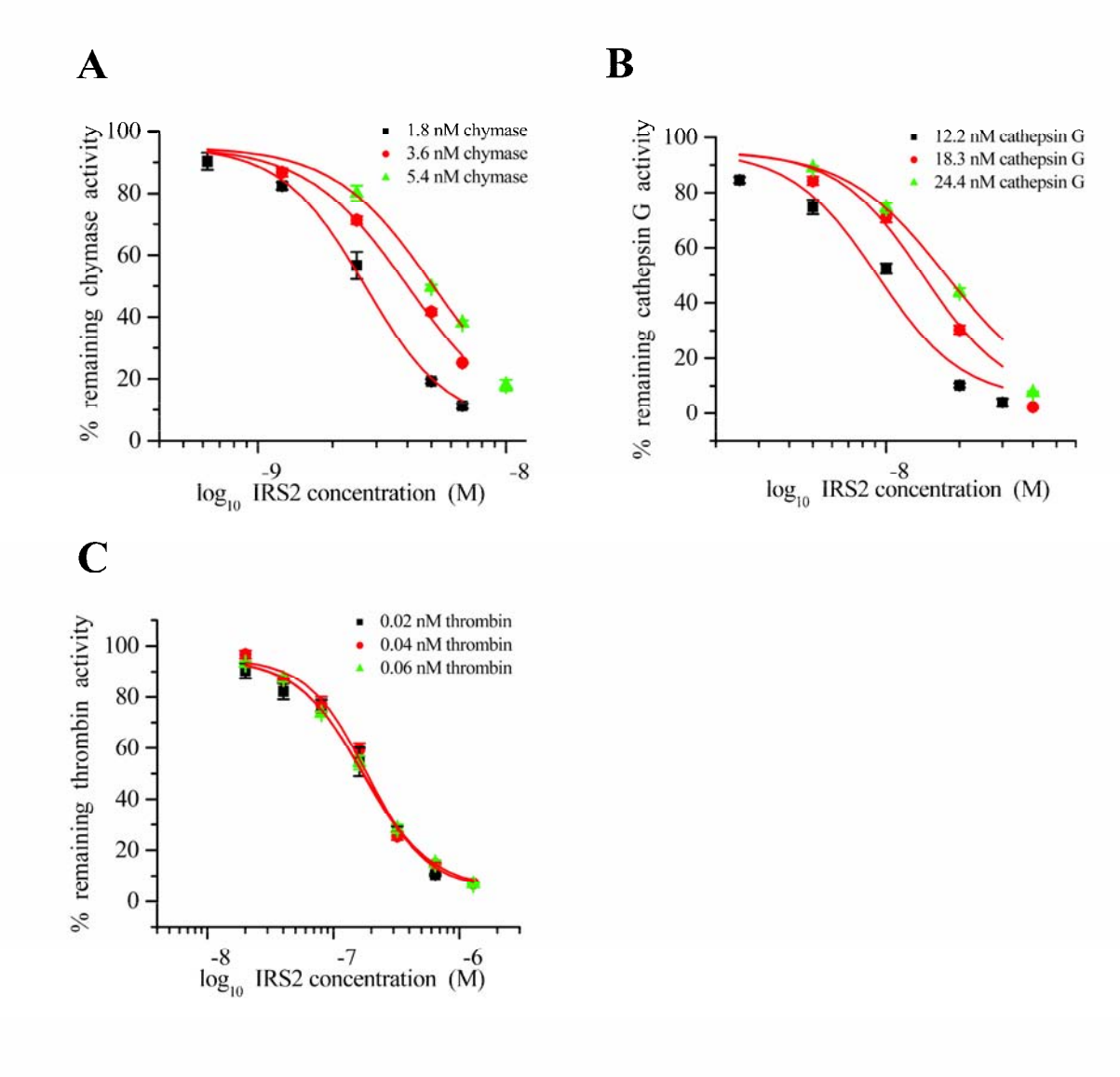

Figure S1. IRS-2 is tight inhibitor of cathepsin G and chymase and classical inhibitor of thrombin. The inhibition curve changed when using different concentrations of chymase (A) and cathepsin G (B).The increase of used enzyme had no effect on the inhibitory profile of IRS-2 in the case of thrombin (C).

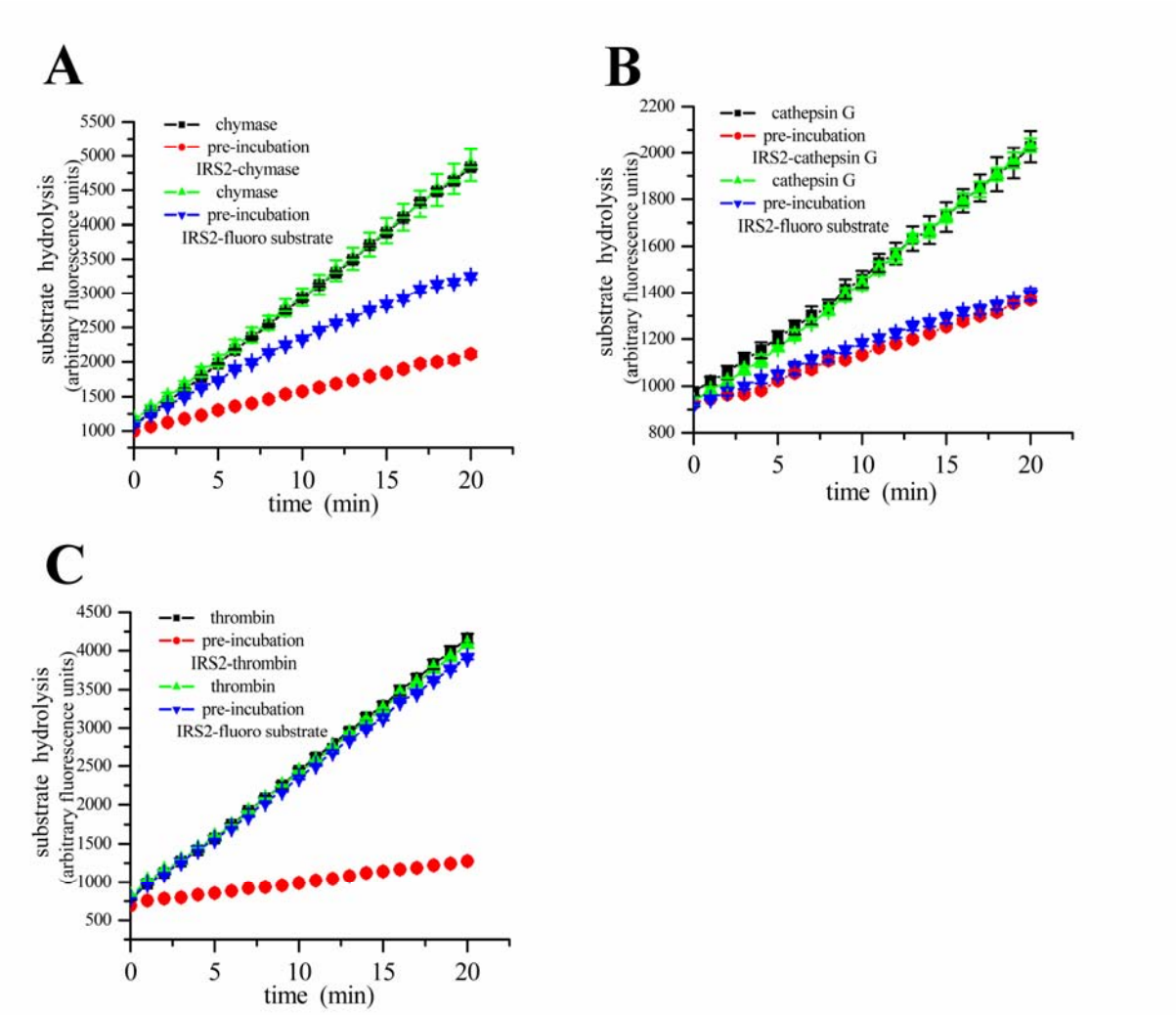

Figure S2. IRS-2 is a fast inhibitor of cathepsin G, slow inhibitor of chymase, which can compete with the substrate for the enzyme and slow inhibitor of thrombin, which cannot compete with the substrate for the enzyme.