

A Novel Serpin with Antithrombin-Like Activity in *Branchiostoma japonicum*: Implications for the Presence of a Primitive Coagulation System

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Abstract

Serine protease inhibitors, or serpins, are a group of widely distributed proteins with similar structures that use conformational change to inhibit proteases. Antithrombin (AT) is a member of the serine protease inhibitor superfamily and a major coagulation inhibitor in all vertebrates, but its evolutionary origin remains elusive. In this study we isolated for the first time a cDNA encoding an antithrombin homolog, *BjATI*, from the protochordate *Branchiostoma japonicum*. The deduced protein BjATI consisted of 338 amino acids sharing 36.7% to 41.1% identity to known vertebrate ATs. BjATI contains a potential N-linked glycosylation site, two potential heparin binding sites and the reactive center loop with the absolutely conserved sequence Gly-Arg-Ser; all of these are features characteristic of ATs. All three phylogenetic trees constructed using Neighbor-Joining, Maximum-Likelihood and Bayesian-Inference methods also placed BjATI together with ATs. Moreover, BjATI expressed in yeast cells was able to inhibit bovine thrombin activity by forming a SDS-stable BjATI-thrombin complex. It also displays a concentration-dependent inhibition of thrombin that is accelerated by heparin. Furthermore, *BjATI* was predominantly expressed in the hepatic caecum and hind-gut, agreeing with the expression pattern of AT in mammalian species. All these data clearly demonstrate that BjATI is an ortholog of vertebrate ATs, suggesting that a primitive coagulation system emerged in the protochordate.

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Introduction

Blood coagulation, or clotting, is of vital importance for the survival of both vertebrates and invertebrates-by preventing the leakage of blood from the sites of injury and impeding infection by the microbial invaders, although the coagulation system of invertebrates is distinct from that of vertebrates [1,2]. It is known that clotting follows the same fundamental pattern in all vertebrates, culminating the thrombin-catalyzed conversion of fibrinogen to fibrin [3,4]. How the vertebrate coagulation system evolved from an entirely dissimilar invertebrate coagulation cascade has been a longstanding issue to biologists. Recently, the jawless fish lampreys have been shown to possess a reduced set of clotting factors observed in higher vertebrates [5], while none of the principal clotting factors are found in the urochordate *Ciona intestinalis* [6]. The basal chordate, amphioxus, as the extant representative of subphylum Cephalochordata, has a heart homolog [7] and a circulation system with a fundamental organization found in all chordates [8,9], providing an ideal model for insights into the origin and evolution of vertebrate coagulation system. Previous studies have shown that amphioxus has plasminogen-like protein [10,11,12] and amphioxus humoral fluid has been shown to cross react with human antithrombin antibody [13]. Bioinformatic approaches to inventory the presence or absence of genes involved in blood coagulation processes supports the view that these systems became progressively more

complex during the period between the divergence of jawless fish and the appearance of mammals. Furthermore, the root of coagulation systems may extend back to protochordates. However, for this evolutionarily important organism, amphioxus, the coagulation system remains largely unclear.

Serine protease inhibitors, or serpins, are a group of widely distributed proteins with similar structures that use conformational change to inhibit proteases. The first members of the serpin superfamily studied extensively were the human plasma proteins antithrombin and antitrypsin [14], which play key roles in controlling blood coagulation and inflammation. Serpin-like genes have been identified in animals, poxviruses, plants, bacteria and archaea. Eukaryotic serpins have been divided into 16 clades [15]. Antithrombins (also known as antithrombin III; ATs) all belong to the members of the clade C serpin superfamily, which consists of a single chain glycoprotein containing 430 to 439 amino acid residues and has a molecular weight of approximately 58 kDa [16]. There are some common features in clade C members, such as potential N-linked glycosylation site, heparin binding sites and the absolutely conserved reactive center loop sequence Gly-Arg-Ser, which makes them different from other serpin members [16]. AT is able to neutralize most serine proteinases in blood such as thrombin and cofactor Xa by forming stable equimolar complexes with the target enzymes [17,18], and is thus a major regulator of the blood coagulation system, playing a crucial role in the maintenance of normal hemostasis [19]. The formation of AT-

proteinase complexes is slow under physiological conditions, but is accelerated markedly by heparin [20]. AT has been identified in several mammalian species such as humans, cow, horse, pig, sheep, rabbit, mouse, rat and hamster [21–25]. It is primarily synthesized in the liver and secreted into plasma [26–28], although production by endothelial cells was also reported [29]. AT has also been documented in some non-mammalian vertebrates like cartilaginous fish, bony fish, amphibians, reptiles and birds [30,16,31,32]. So far, ATs have been identified only in vertebrates, and its emergence during animal evolution remains elusive. The purposes of this study was therefore to determine if the *AT-like* gene (designated *BjAT1*) is present in the chordate amphioxus *Branchiostoma japonicum*, and if so, to examine its characteristics and expression pattern, and to test if it is functionally similar to vertebrate AT.

Materials and Methods

Cloning and sequencing of AT-like cDNA

All animal experiments were carried out in accordance with the guidelines of the Laboratory Animal Administration Law of the People's Republic of China, with the permit number SD2007695 approved by the ethics committee of the Laboratory Animal Administration of Shandong province.

Total RNAs were extracted with Trizol (Invitrogen) from *B. japonicum* collected in the vicinity of Qingdao, China, and polyA⁺ RNA was purified using polyA tract mRNA isolation system II (Promega) according to the manufacturer's instructions. The first-strand cDNA was synthesized with the reverse transcription system (Promega) using oligo d(T) primer. The fragments of *B. japonicum* AT-like cDNA, *BjAT1*, were amplified by PCR with degenerate primer pairs, S1 and A1 (Table 1), which were designed based on the sequences of conserved motifs of vertebrate ATs. The PCR amplification was carried out at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 51.6°C for 30 s, 72°C for 90 s and the final extension step at 72°C for 7 min. PCR products were purified and re-amplified. A 988 bp fragment was subcloned and sequenced. The gene-specific primers S2 and A2 (Table 1) were used in RACE (rapid amplification of cDNA ends) reactions for full-length cDNA synthesis, according to the instructions of SMARTTM RACE cDNA amplification kit (Clontech).

Sequence analysis

The deduced amino acid sequence was analyzed with the BLAST algorithm at NCBI web site and SWISS-MODEL server at the Expert Protein Analysis System (<http://www.expasy.org/>). Multiple alignments were performed using ClustalX 1.81 (Thompson et al., 1994) and Multiple Alignment show program

(<http://www.biosoft.net/sms/index.html>). Identity score was obtained using DNASTAR software package by Clustal method [33]. Using ClustalX-aligned amino acid sequences, Neighbor-Joining (NJ) tree, Maximum-Likelihood (ML) tree and Bayesian-Inference (BI) tree were constructed. Statistical supports in the NJ tree was represented by percentage of 1000 bootstrap replicates with distances computed by JTT Matrix model in MEGA4.0 [34]. For ML tree, ProtTest 1.4 [35] was used to determine the best protein substitution model and estimate the gamma parameters. After running ProtTest 1.4, the ML tree was constructed using phyML (<http://atgc.lirmm.fr/phyml/>) by the LG+I+G+F model. In addition, a BI tree was constructed using MrBayes 3.12 [36]. All the sequences used here are listed in Table S1.

The tertiary structure of *BjAT1* was predicted with a homology-modeling method via ESyPred3D using neural networks, using human AT as template [37]. The visualization and characterization of the three-dimensional structures of the human AT and *BjAT1* were performed with software PyMOL [38].

Preparation of anti-BjAT1 antibody

The complete coding region of *BjAT1* was amplified by PCR with the primer S3 and A3 (Table 1), and sub-cloned into the EcoRI/XhoI site of the pET28a (Novagen) to generate the expression construct pET28a/*BjAT1* with an N-terminal His tag. *Escherichia coli* BL21 transformation and isopropyl β-D-thiogalactoside (IPTG) inducing procedures followed the methods specified by the manufacturer (Novagen). *BjAT1* expressed in *E. coli* was purified using a Ni-NTA resin column (Novagen) according to the manufacturer's protocols. Approximately, 2 mg of the purified *BjAT1* protein was emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites in rabbits. Three booster injections of 1 mg antigen mixed with Freund's incomplete adjuvant were administered subcutaneously at intervals of 2 weeks. Eight days after the final booster, blood was collected and serum prepared. The antiserum was aliquoted and stored at -70°C until used.

Expression of BjAT1 in Pichia pastoris

The complete coding region of *BjAT1* cDNA was amplified by PCR with specific primers S3 and A4 (Table 1). The PCR product was digested with EcoRI and XbaI, and sub-cloned into the plasmid expression vector pPICZαA (Invitrogen) previously cut with the same restriction enzymes. The identity of the insert was verified by sequencing, and the plasmid was designated pPICZαA/*BjAT1*.

The constructed plasmid pPICZαA/*BjAT1* was linearized with SacI and transformed into the competent cells of *P. pastoris* X33 by electroporation as recommended by manufacturer's instructions

Table 1. Sequences of the primers used in this study.

Primer	Sequence (5'-3')	Sequence information
S1 (sense)	TCTTCTCWCBBTACAGYATCTC	<i>BjAT1</i> cDNA fragment primer
A1 (antisense)	TGDATRAAGAAVAGGAACGG	<i>BjAT1</i> cDNA fragment primer
S2 (sense)	CGAAGCTTTGTTGGACGCCACACGAGG	3'RACE primer
A2 (antisense)	TTGCCTCCACCAGTGTGTCTTGTTC	5'RACE primer
S3 (sense)	CCGGAATTCATGGCCATGACATACATGG	Recombinant primer
A3 (antisense)	CCGCTCGAGATTACTCATTGGGTTGGTC	Recombinant primer
A4 (antisense)	CTAGTCTAGAGGCTCATTGGGTTGGTACC	Recombinant primer

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(Invitrogen). One positive clone was selected and incubated into 100 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-4} mg/ml biotin and 1% glycerol) and grown at 28°C until the culture reached $OD_{600} = 2-6$. The cells were harvested by centrifuging at 2,000 g for 10 min at room temperature, re-suspended in 500 ml BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-4} mg/ml biotin and 0.5% methanol) and cultured at 28°C. To induce expression, methanol was added every 24 h to a final concentration of 0.5% for two successive days. The culture was centrifuged at 10,000 g for 20 min at 4°C. Subsequently, solid $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 75% saturation. After stirring at 4°C overnight, the suspension was centrifuged at 10,000 g for 20 min at 4°C. The precipitate was suspended in dialysis buffer (20 mM PBS with 500 mM NaCl, pH 7.4), and dialyzed against the same buffer, which was changed 3 to 4 times, until trace of $(NH_4)_2SO_4$ was removed. The dialyzed sample was pooled, filtered through a 0.45 μ m Millipore filter, and loaded onto a Ni-NTA resin column (Amersham). The column was washed with the washing buffer (20 mM PBS containing 500 mM NaCl and 20 mM imidazole, pH 7.4) and eluted with the elution buffer (20 mM PBS containing 500 mM NaCl and 250 mM imidazole, pH 7.4). The eluted sample was concentrated and solvent exchanged to 50 mM Tris-HCl (pH 7.6) by using Amicon Ultra-15 (MILLIPORE). The purity of the recombinant BjATI was analyzed by a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [39], and stained with Coomassie brilliant blue R-250. The recombinant BjATI was aliquoted and stored at -70°C until used. The protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard [40].

Western blotting

The recombinant BjATI expressed in *P. pastoris* was run on a 12% SDS-PAGE gel under reducing condition. The gel was washed for 15 min in 20 mM PBS containing 0.1% Tween-20, and the proteins on the gel were blotted onto PVDF membrane (Amersham). The blotted membranes were incubated in 20 mM PBS containing 3% defatted milk powder at 30°C for 2 h, and then in the anti-BjATI serum diluted 1:500 with 20 mM PBS containing 0.1% Tween-20 for 2 h, or in the anti-His antibody (TIANGEN) diluted 1:1,000 with the same solution. After washing in 20 mM PBS, the membranes were incubated in horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Zhongshan, China) diluted 1:1,000 at 30°C for 2 h. The bands were visualized using DAB and 0.03% H_2O_2 .

Assay for AT-like activity

The activity, if any, of the recombinant BjATI expressed in *P. pastoris* was detected by a chromogenic assay using Actichrome AT III kit (American Diagnostica Inc., Stamford). In this two stage method, 2.5 nkat of bovine thrombin was mixed with 200 μ l of 50 mM Tris-HCl (pH 7.6) containing 1.92 μ g of BjATI in the presence or absence of 1.8 U/ml heparin. Meanwhile, 2.5 nkat of bovine thrombin was mixed with 200 μ l of BjATI solutions that each contained 3.2, 9.6 and 16 μ g/ml BjATI, respectively, or with 200 μ l of diluted human standard plasma, in the presence of excess of heparin (1.8 U/ml). After initial incubation at 28°C for 30 min, the thrombin-specific chromogenic substrate, Spectrozyme TH, was added to the mixtures, giving a final concentration of 0.18 μ M, and incubated at 37°C for 1 min. After addition of 200 μ l of acetic acid to terminate the reaction, the residual thrombin activity was determined by measuring the absorbance at

405 nm under a microplate spectrophotometer (GENios Plus Tecan). The inhibitory ability of BjATI on thrombin was inversely proportional to the residual thrombin activity.

Assay for formation of BjATI-thrombin complex

The purified recombinant BjATI expressed in *P. pastoris* was incubated with bovine thrombin (molecular mass ~ 34 kDa) in 50 mM Tris-HCl (pH 7.6) containing excess of heparin (1.8 U/ml), at a molecular ratio 1:1 at 28°C for 30 min. The reaction products were separated by reducing SDS-PAGE (8%) and immunostained as described above. The humoral fluid was prepared by the method of Wang et al. [41]. Briefly, about 1000 amphioxus were rinsed with distilled water, wiped out thoroughly with sterilized water, and then cut into about 2 mm^3 pieces on ice to bleed. After centrifugation at 12,000 g at 4°C for 30 min, the supernatant was collected and stored at -70°C until used. Diluted humoral fluids (50 μ l; 15 mg proteins/ml) was incubated with bovine thrombin (100 μ g) in order to test the presence of native BjATI in *B. japonicum*.

Northern blotting and In situ hybridization histochemistry

Total RNA was extracted with Trizol (Gibco) from the adult amphioxus *B. japonicum* ground in liquid nitrogen. An aliquot of 5 μ g RNAs were each electrophoresed and blotted onto a Nylon membrane (Roche, Germany). The digoxigenin (DIG)-labeled BjATI riboprobes of about 1000 bp were synthesized *in vitro* from linearized plasmid DNA following the DIG-UTP supplier's instructions (Roche, Germany). Northern blot analysis was carried out as described previously [42].

The sexually-matured amphioxus were cut into 3 to 4 pieces and fixed in freshly prepared 4% paraformaldehyde in 100 mM phosphate buffered saline (PBS; pH 7.4) at 4°C for 8 h. The samples were dehydrated, embedded in paraffin, and sectioned at 6 μ m. The sections were mounted onto poly-L-lysine coated slides, dried at 42°C for 36 h, and de-paraffinized in xylene for 20 min (two changes for 10 min each), followed by immersion in absolute ethanol for 10 min (two changes for 5 min each). They were re-hydrated, and equilibrated in double distilled H_2O containing 0.1% DEPC. The digoxigenin (DIG)-labeled BjATI riboprobes of about 500 bp were synthesized *in vitro* from linearized plasmid DNA following the DIG-UTP supplier's instructions (Roche). *In situ* hybridization histochemistry was carried out as described by Fan et al. [42].

Results

Sequence and phylogeny of BjATI

A cDNA fragment of approximately 988 bp containing the domain SERPIN was obtained from *B. becheri* by PCR using the degenerate primer pair S1 and A1. The sequences of these primers were designed based on the conserved domain from known antithrombin sequences. Based on the partial cDNA sequence, the primers for 3'RACE and 5'RACE, S2 and A2, were designed, and two cDNA fragments of 1339 bp and 259 bp in length were produced by PCR using S2 and A2, respectively. The full-length cDNA of BjATI was assembled by overlapped cDNA fragments, and was deposited in GenBank (accession number: **EU164803**). The cDNA was 1943 bp long, and included an open reading frame (ORF) of 1017 bp, a 5'-untranslated region (UTR) of 29 bp and a 3'-UTR of 897 bp. The initiation codon, ATG, was in accordance with Kozak rule (A/GXXATGG), and the 3'-UTR had the polyadenylation signal AATAAA. The ORF coded for a deduced protein of 338 amino acids. There was a potential N-

linked glycosylation site located at the residual position N33, but it lacked a signal peptide at its N-terminus as predicted by the Signal IP 3.0 server [43].

Blastp searching at NCBI revealed that BjATI had the conserved domain SERPIN at residues 1–336, and shared 38.2%, 36.7%, 38.5%, 41.1%, 39.1%, 39.6%, 39.6%, 41.7%, 38.5% and 40.8% identity to the antithrombins from fugu, salmon, zebrafish, frog, turtle, tuatara, chicken, ostrich, cow and humans, respectively (Fig. 1). Also, BjATI shared ~40% identity with some serpin clade B members, such as Bovine SCCA (XP001254097), Bovine PI-6 (O02739) and Human SCCA (P29508). The predicted 3D structures of human AT and BjATI are shown in Figure 2. Although the numbers of β -sheets at N-termini (BjATI had 3 β -sheets, while human AT had 6 β -sheets) and glycosylation sites in human AT and BjATI were different, their general 3D structures show significant similarity. Moreover, the reactive side loop region of BjATI was closely resembles that of human AT.

Sequence comparison showed that BjATI contains a reactive center loop (RCL) similar to that of ATs. The RCL forms an extended and exposed conformation above the body of AT scaffold, and is responsible for the interaction with target proteases. The 20 amino acid residues constituting the RCL are numbered Pn- ... -P1-P1'- ... -Pn', where P1-P1' is ultimately cleaved [44]. The residues P2, P1 and P1' with the sequence Gly-Arg-Ser, the primary determinants of AT specificity, were absolutely conserved in BjATI and other ATs (Fig. 3). Besides, the P8 (Thr) and P10 (Ala), which are important for the formation of covalent complex with target proteinase, were also strictly conserved in BjATI and other ATs. Comparisons to human antithrombin shows BjATI contains the potential heparin binding site residues H120 and K136 (numbering as human AT; [16]) although it did not contain the heparin binding site residues K11, R13, R46, R47, K125, R129, R132 and K133. Interestingly, in BjATI the K125 is replaced by asparagine (an N-linked

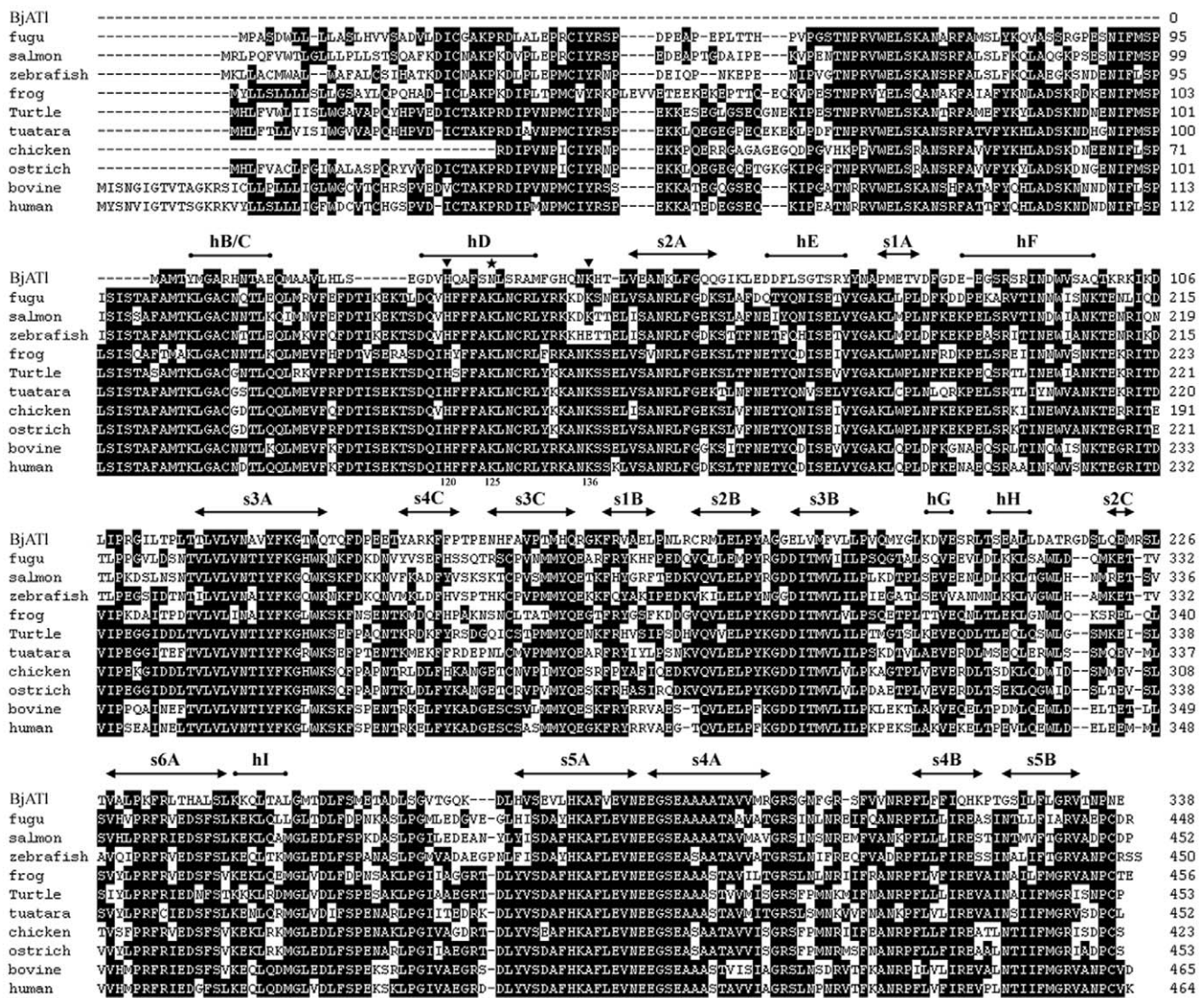


Figure 1. Aligned sequences of BjATI and 10 vertebrate antithrombins. Mouse antithrombin numbering is used. Secondary structural elements of BjATI predicted based on the structure of human antithrombin are shown above the sequences. Solid arrows indicate β -sheet, cylinders represent α -helices, triangles show the heparin-binding sites, and star indicates potential glycosylation site. Amino acid residues that are conserved in at least 50% of sequences are shaded in dark.

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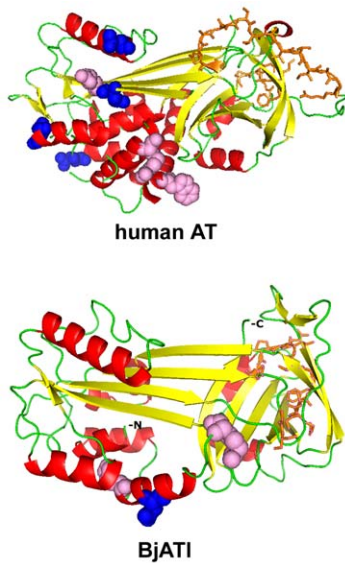


Figure 2. Cartoon representation of homology models of the human AT (A) and BjATI (B). α -helix residues are colored with red, β -sheet residues with yellow, and loop and unassigned residues with green. Pink spheres show the heparin-binding sites, and blue spheres indicate the potential glycosylation site. Orange sticks show the RCL (reactive center loop) region.
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glycosylation site) (Fig. 1), which may also play a crucial role in heparin binding [45].

Among the 16 clade serpins, BjATI shared high sequence identity with clade B and clade C serpins. The clade B serpins lack the signal peptide, are primarily intracellularly localized, and are supposedly the ancestors to the majority of extracellular serpin proteins (including ATs) [46]. Like clade B members, BjATI does not have signal peptide. In contrast, the residues at P2, P1 and P1' of BjATI are different from clade B members; they are Gly-Arg-Ser, which are absolutely conserved in and typical of ATs (Fig. 3). Both clade B and clade C serpins were included in the phylogenetic tree construction. As shown in Figure 4, all the phylogenetic trees constructed by different methods revealed that BjATI was clustered together with ATs, and located at the root of antithrombin (clade C serpin) branch, separating from clade B serpin members. These indicated that BjATI is an ortholog of antithrombins (clade C serpin).

Expression of BjATI in yeast cells

The constructed plasmid pPICZ α A/BjATI was linearized with SacI and transformed into *P. pastoris* X33. The positive clones were screened and utilized for expression. The recombinant protein with the His-tag was purified by affinity chromatography on a Ni-NTA resin column, and analyzed by a 12% SDS-PAGE, followed by staining with Coomassie Brilliant Blue R-250, which demonstrated the presence of a single protein band of approximately

	P15	P14	P13	P12	P11	P10	P9	P8	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'
Clade C	BjATI	G	S	E	A	A	A	T	A	V	V	M	R	G	R	S	G	N	F
	fugu AT	G	S	E	A	A	A	T	A	A	V	A	T	G	R	S	I	N	L
	salmon AT	G	S	E	A	A	A	T	A	V	M	A	V	G	R	S	I	N	S
	zebrafish AT	G	S	E	A	S	A	T	A	V	V	A	T	G	R	S	L	N	I
	frog AT	G	S	E	A	A	A	S	T	A	V	I	L	T	G	R	S	L	N
	turtle AT	G	S	E	A	A	A	S	T	V	V	M	I	S	G	R	S	F	P
	tuatara AT	G	S	E	A	A	A	S	T	A	V	M	I	T	G	R	S	L	S
	chicken AT	G	S	E	A	S	A	T	A	V	V	I	S	G	R	S	F	P	M
	ostrich AT	G	S	E	A	S	A	T	A	V	V	I	S	G	R	S	F	P	M
	bovine AT	G	S	E	A	A	A	S	T	V	I	S	I	A	G	R	S	L	N
	human AT	G	S	E	A	A	A	S	T	A	V	V	I	A	G	R	S	L	N
	human MNEI	G	T	E	A	A	A	T	A	G	I	A	T	F	C	M	L	M	P
	pig MNEI	G	T	E	A	A	A	T	A	G	I	A	V	F	A	M	L	M	P
	horse MNEI	G	T	E	A	A	A	T	A	G	T	I	M	L	A	M	L	M	P
	dog SCCA	G	T	E	A	A	A	S	T	D	V	E	V	I	A	K	S	A	P
	bovine SCCA	G	T	E	A	A	A	A	T	G	V	V	F	T	R	T	S	L	P
	human SCCA	G	A	E	A	A	A	A	T	A	V	V	G	F	G	S	S	P	T
Clade B	mouse PI-6	G	T	E	A	A	A	T	A	G	M	M	T	V	R	C	M	R	F
	human PI-6	G	T	E	A	A	A	T	A	A	I	M	M	M	R	C	A	R	F
	human PI-8	G	T	E	A	A	A	T	A	V	V	R	N	S	R	C	S	R	M
	rat Maspin	G	G	D	S	I	E	V	P	G	S	R	I	L	Q	H	K	D	E
	chicken OVAL	G	R	E	V	V	G	S	A	E	A	G	V	D	A	A	S	V	S
	human PAI-2	G	T	E	A	A	A	G	T	G	G	V	M	T	G	R	T	G	H
	mouse PI-9	G	T	E	A	A	A	S	A	I	I	E	F	C	C	A	S	S	V

Figure 3. Comparison of serpin RCLs. Clade C (upper panel) and Clade B (lower panel) serpin RCLs from P15-P4' were aligned. Residues from P2, P1 and P1' are framed as box, and the residues absolutely conserved are shaded in dark. The strictly conserved residues at P8 and P10 are shaded in grey.
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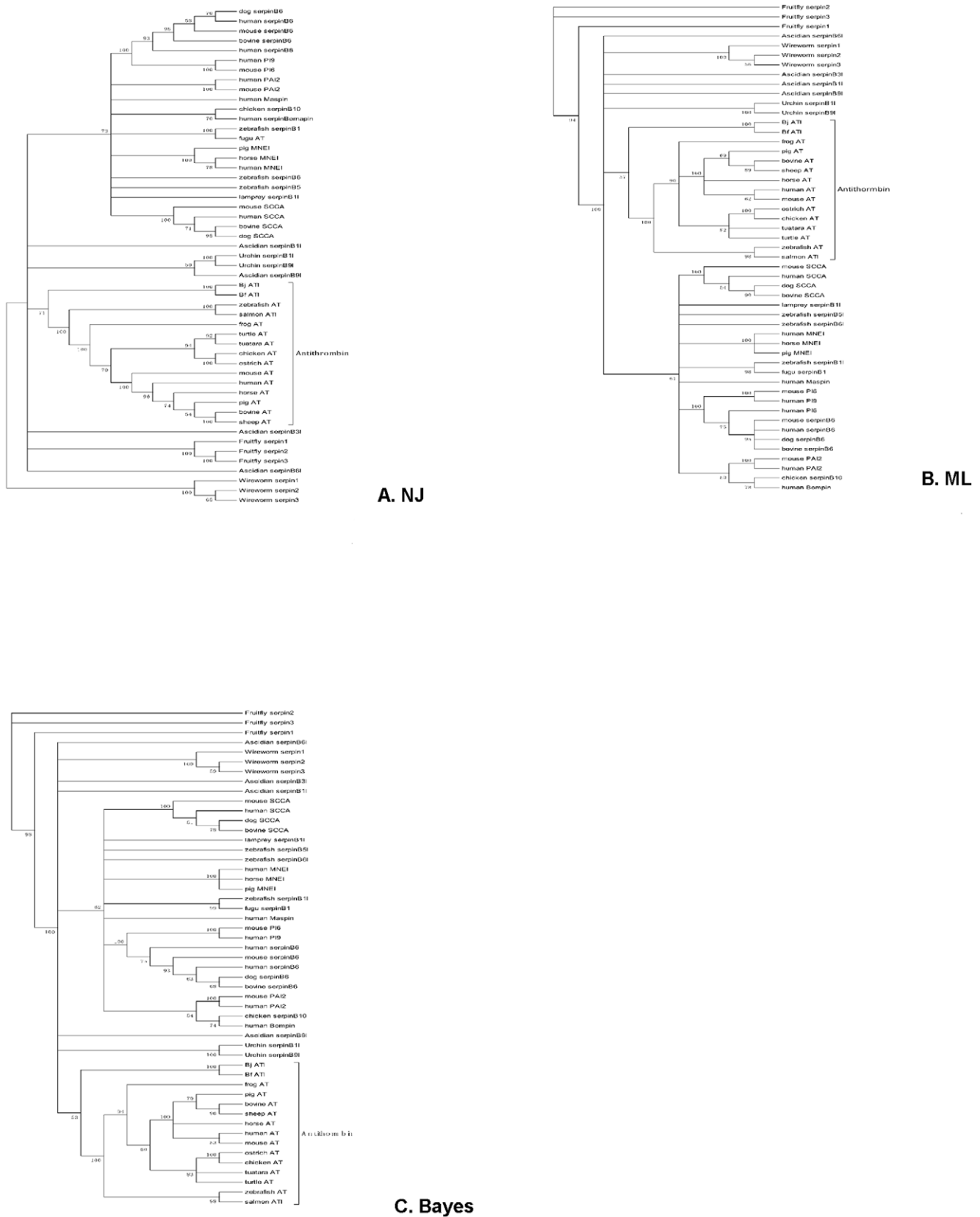


Figure 4. The phylogenetic trees constructed using the sequences of BjATI and other representative members of serpin cladeB and cladeC. (A) The neighbor-joining (NJ) tree constructed using the package MEGA4.0; (B) The maximum likelihood (ML) tree using the program PhyML3.0; and (C) The Bayesian inference (BI) tree using MrBayes3.04b. Branches with bootstrap value <50% are cut off. Accession numbers for the sequences used are listed in Table S1. doi:10.1371/journal.pone.0032392.g004

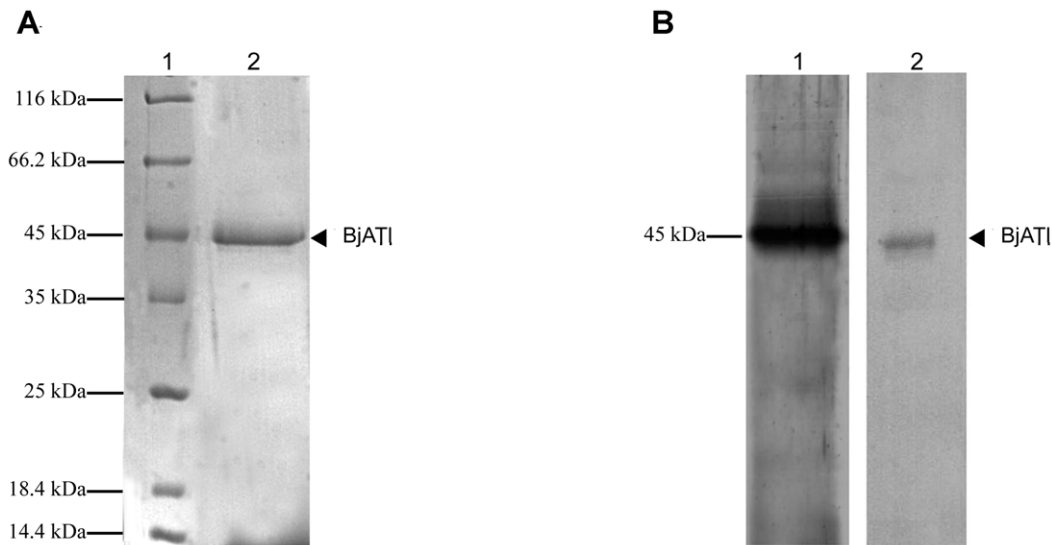


Figure 5. SDS-PAGE and Western blotting of recombinant BjATI expressed in *Pichia pastoris*. (A) SDS-PAGE of recombinant BjATI purified on Ni-NTA resin column. Lane 1, molecular mass standards; Lane 2, recombinant BjATI. (B) Western blotting. Lane 1, the supernatant of *Pichia pastoris* with *BjATI* insertion induced with methanol, and immunostained with anti-BjATI antiserum; Lane 2, the supernatant of *Pichia pastoris* with *BjATI* insertion induced with methanol, and immunostained with anti-His tag antiserum.
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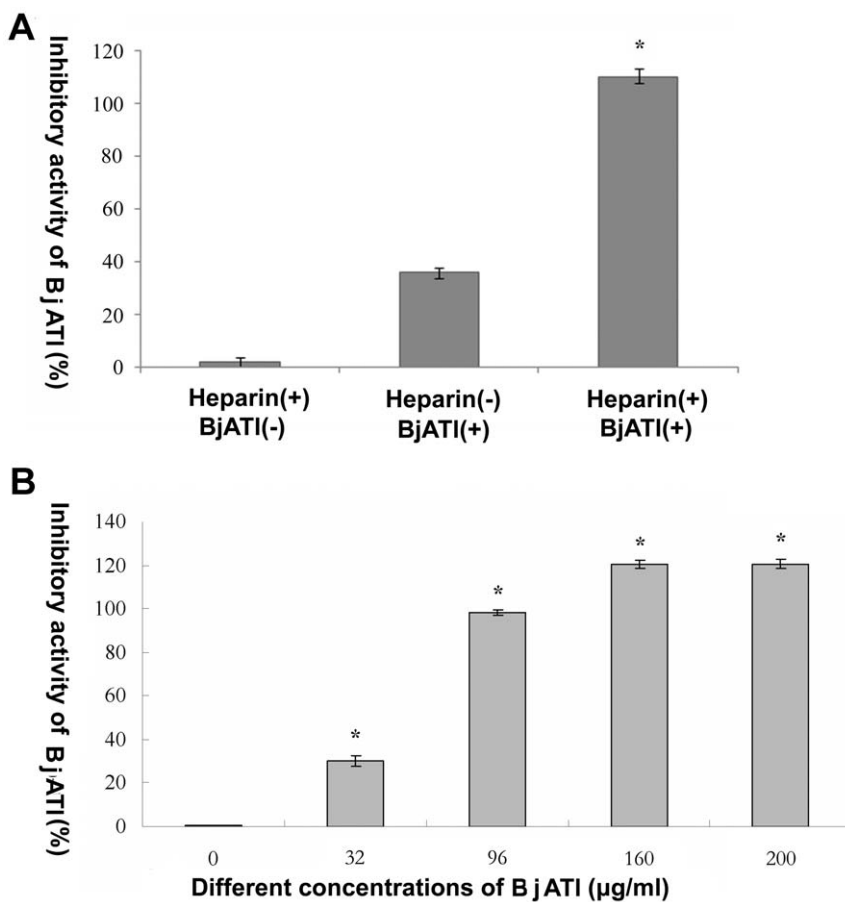


Figure 6. Inhibitory activity of recombinant BjATI. (A) The inhibitory activity of recombinant BjATI in the presence (+) or absence (-) of heparin. (B) The inhibitory activity of different concentrations of recombinant BjATI in the presence of heparin. The inhibitory activity of BjATI was determined for each group and values were shown as means \pm SD (n=3). Significant differences ($p < 0.001$) are indicated by an asterisk (*).
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45 kDa (Fig. 5A). Western blotting revealed that the purified protein reacted with both rabbit anti-BjAT1 serum and anti-His-tag antibody, indicating that BjAT1 was correctly expressed (Fig. 5B).

Inhibitory effect of BjAT1 on bovine thrombin activity

The inhibitory activity of BjAT1 was quantified by comparison to a standard curve prepared with diluted normal human plasma. By definition, AT activity of diluted normal plasma is 100%. As shown in Figure 6, BjAT1 was capable of inhibiting bovine thrombin activity in a concentration-dependent manner, and its inhibitory activity was significantly accelerated by heparin.

BjAT1 forms SDS-stable complex with thrombin

To detect the interaction between BjAT1 and thrombin, BjAT1 was exposed to bovine thrombin. Pilot experiments showed that anti-BjAT1 serum reacted with BjAT1, forming a single band of ~45 kDa, whereas it was not reactive with bovine thrombin (Fig. 7A). Western blotting revealed that the incubation of bovine thrombin with recombinant BjAT1 resulted in the formation of a SDS-stable complex (Fig. 7B), which had a molecular mass of ~80 kDa (BjAT1-thrombin complex). Another protein band was observed to migrate slightly faster than the residual non-reacted BjAT1, which is apparently the cleaved BjAT1 as reported by Mochizuki et al [47]. Similarly, the incubation of bovine thrombin with *B. japonicum* humoral fluids led to the occurrence of two major bands at ~45 kDa and ~80 kDa (Fig. 7B), suggesting the presence of native BjAT1 protein in *B. japonicum*, which can interact with thrombin.

Tissue-specific expression of BjAT1 in adult amphioxus

Northern blotting revealed the presence of an approximately 2000 bp transcript in *B. japonicum* (Fig. 8). To explore the expression pattern of *BjAT1* in adult *B. japonicum*, tissue section *in situ* hybridization was conducted and the results demonstrated that *BjAT1* transcript was most abundant in the hepatic caecum and hind-gut, and at a lower level present in the gill and ovary, while it was absent in the epidermis, muscle, neural tube, notochord and testis (Fig. 9), implicating a tissue-specific expression pattern of *BjAT1* in adult *B. japonicum*.

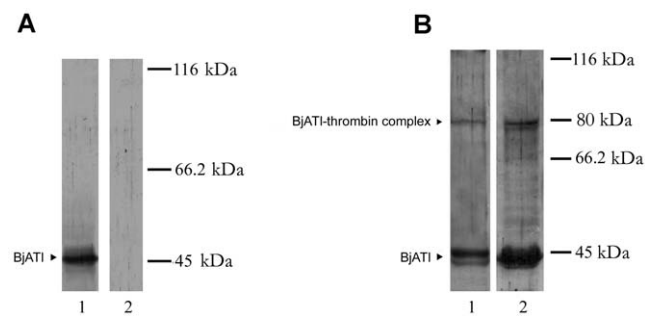


Figure 7. Analysis of complex formation with thrombin. Purified BjAT1 or amphioxus humoral fluids were incubated with bovine thrombin. After SDS-PAGE (8% gels) under reducing condition, the reaction products were immunostained with anti-BjAT1 antiserum. (A) Lane 1, purified BjAT1; Lane 2, bovine thrombin. (B) Lane 1, purified BjAT1 incubated with bovine thrombin; Lane 2, amphioxus humoral fluids incubated with bovine thrombin. The positions and molecular masses of marker proteins are indicated on the right. doi:10.1371/journal.pone.0032392.g007

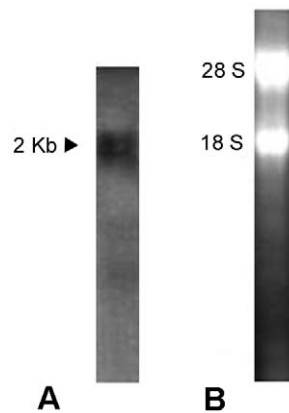


Figure 8. Northern blotting. (A) The blot was hybridized with Dig-labeled BjAT1 RNA probe. The arrow indicates the position of molecular size equivalent to 2000 bp. (B) A total of 5 µg RNA was analyzed in 1.2% agarose formaldehyde-denaturing gel. doi:10.1371/journal.pone.0032392.g008

Discussion

Previous studies have shown the presence of AT in jawed vertebrates [1], while it was recently found that a putative AT-like homolog is present in amphioxus *B. japonicum* [12]. Here we demonstrate for the first time a novel member of serpin family with AT-like activity in *B. japonicum*. The deduced 338 amino acids long protein, BjAT1, shares more than 36.7% identity to known ATs and contains the conserved domain SERPIN at residues 1–336 (including the RCL with the conserved AT specific sequence GRS), an N-linked glycosylation site and the potential two heparin binding sites. Additionally, the recombinant BjAT1 exhibits thrombin-inhibiting activity, which can be enhanced by heparin. Mammalian antithrombin inactivates the coagulation protease thrombin by forming stable equimolar AT/target enzyme complex [48,49]. BjAT1 is also able to interact with bovine thrombin in the presence of heparin by forming BjAT1-thrombin complex (Fig. 7B), suggesting that BjAT1, like mammalian AT, utilizes a similar mechanism to bind to thrombin. Both sequencing and functional data clearly indicate that BjAT1 is a novel member of serpin with some AT-like activity. Previously, plasminogen-like protein has been identified in amphioxus [13]. Taken together, these findings appear to provide us a clue that a primitive coagulation system already emerged in the protochordate.

Clade B serpins lack signal peptide and reside primarily within cells, most members are normally shorter (350–400 amino acids [50]) than ATs. These Clade B serpins are presumed to be ancestors of the majority of extracellular serpins (including antithrombins) [46]. It is of interest to note that BjAT1 shares ~40% identity with some clade B members. Also, all the three phylogenetic trees show that BjAT1 groups at the root of clade C (ATs) branch. It is likely that BjAT1 is the common ancestor of clade B and clade C serpins. These members of the serpin family currently present in mammals, avians and amphibians may have evolved through intragenic duplication and N-terminal amino acid replacement of the protease domain, gene duplication, and exon shuffling and deletion.

Several clade B serpins were found to exist in both intracellular and extracellular forms [46,51]. Western blotting results reveal that BjAT1 is secreted and circulates in the humoral fluids at low levels. This also suggests that the molecular weight of native BjAT1 is approximately 45 kDa, which is closely similar to recombinant BjAT1. As the recombinant BjAT1 used here is expressed in *P. pastoris* X33, and this eukaryotic expression system has the

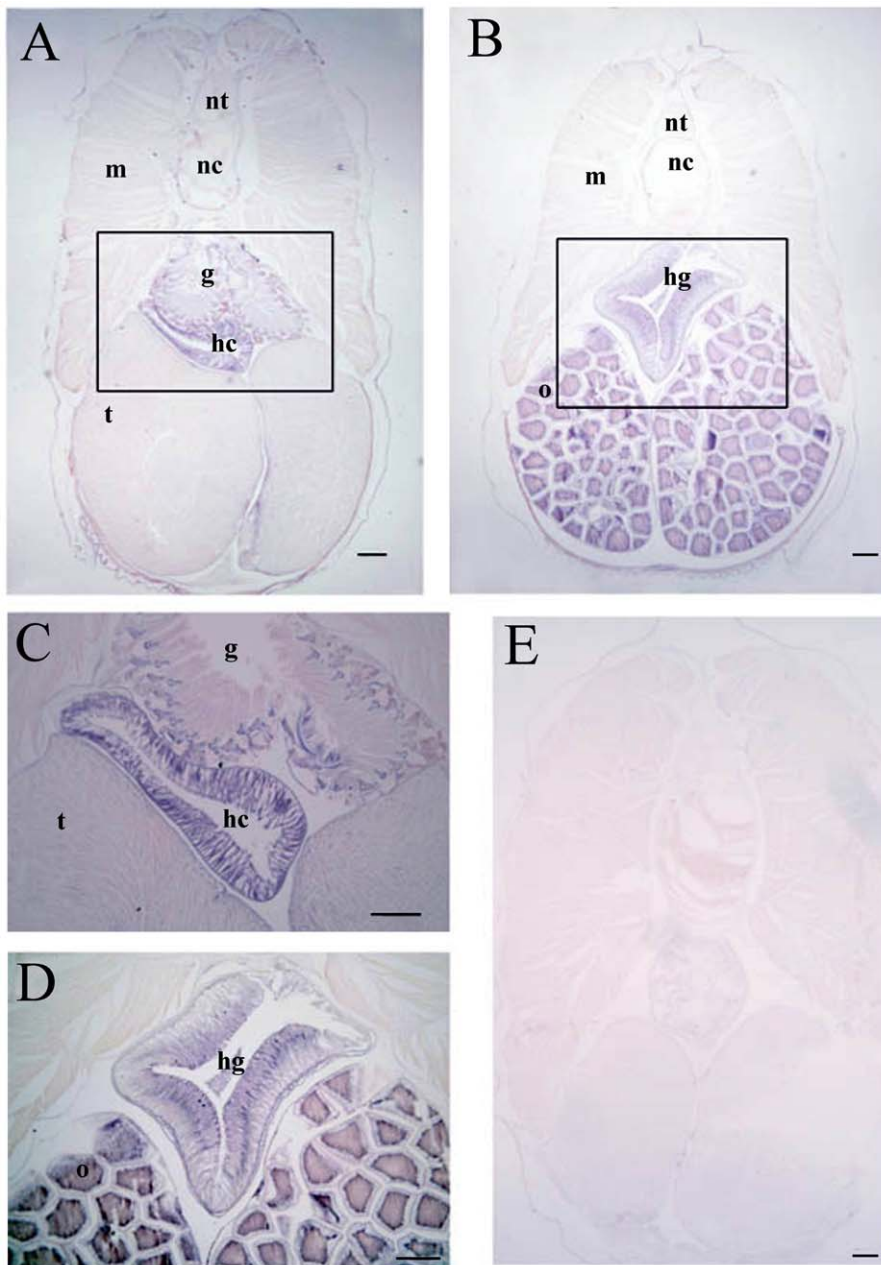


Figure 9. Localization of *BjATI* transcripts in different tissues of adult amphioxus detected by *in situ* hybridization histochemistry. (A) A low magnification section of a male amphioxus showing the presence of *BjATI* mRNA was most abundant in hepatic caecum (hc) and at a lower level present in gill (g). No signal was found in testis (t), muscle (m), notochord (nc) and neural tube (nt). (B) A low magnification section of a female amphioxus showing the presence of *BjATI* mRNA was most abundant in hind-gut (hg) and at a lower level present in ovary (o). (C) and (D) The enlargement of the boxes in A and B. (E) Micrograph showing the absence of *BjATI* transcripts in control section. Scale bars represent 100 μ m. doi:10.1371/journal.pone.0032392.g009

advantage that allows protein glycosylation to take place, it is therefore possible that the function of recombinant BjATI is a partial reflection to native BjATI. It is of note that the molecular mass of BjATI is smaller than that estimated from Liang's study [12]. The reason for this difference is not clear at present, and needs to be clarified in the future.

The liver is the major synthesis site of AT in vertebrates [27–29]. Amphioxus has a hepatic caecum, the pouch that protrudes forward as an outpocketing of the digestive tube and extends along the right side of the posterior part of the pharynx, which has long been considered to be the homologous structure to vertebrate liver

[52–54]. Our study reveals that BjATI exhibits a tissue-specific expression pattern in *B. japonicum*, with the most abundant expression in the hepatic caecum and hind-gut. Broadly speaking, this supports that the homology of the hepatic caecum of amphioxus to the vertebrate liver.

In summary, the present study demonstrates molecularly and functionally the presence of a novel member of serpins with AT-like activity in amphioxus *B. japonicum*, pushing the evolutionary origin of this protein to the invertebrate chordate. This suggests that a primitive coagulation system already emerged in the protochordate.

Supporting Information

Table S1 The names and accession numbers of serpins. (DOC)

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Author Contributions

Conceived and designed the experiments: YC SZ. Performed the experiments: YC. Analyzed the data: YC BG. Contributed reagents/materials/analysis tools: CF YL. Wrote the paper: YC SZ.