- Government should monitor and discourage burning of crop residue through incentives and technology transfer and utilization.

Residue burning in the RWS due to the use of combines has resulted in pollutant emission, loss of nutrients, diminished soil biota, and reduced total N and C in the topsoil layer. The gaseous emissions have been estimated to be 110, 2306, 2 and 84 Gg respectively, for CH_4, CO, N_2O and NO_x from rice and wheat straw burning in India in 2000, which is a noticeable increase in comparison to 1994. There is need to review and upgrade the technology involved with mechanized harvesters, for sustainable utilization of residue thereby overcoming the compulsion to burn residue in the rice–wheat cropping system, the major concern being the short time between harvesting of rice and planting of wheat. Long-term studies on residue incorporation, investigation on resource depletion and related environmental and rural sustainability are required.

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Characterization and expression of *AmphiCB* encoding a cathepsin B proteinase from amphioxus *Branchiostoma belcheri tsingtauense*

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In this study, an amphioxus cDNA, *AmphiCB,* encoding cathepsin B proteinase was isolated from the gut cDNA library of *Branchiostoma belcheri tsingtauense*. It has an open reading frame encoding a precursor protein which consists of 333 residues, including a signal peptide, a pro-peptide and a mature proteinase. The mature *AmphiCB* has all conserved structures characteristic of cathepsin B. The phylogenetic tree constructed shows that *AmphiCB* appears more closely related to invertebrate cathepsin B. Northern blotting and RT–PCR demonstrate that *AmphiCB* transcript was present in all tissues examined, with much stronger expression in the hind-gut and hepatic caecum. This suggests that *AmphiCB* is possibly actively involved in food digestion, in addition to its housekeeping role.

CATHEPSINS are lysosomal cysteine proteinases belonging to the papain superfamily (C1A), which have been classified

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into two structurally distinct families: enzymes with the highly conserved interspersed ERFNIN motif in the propeptide region, such as cathepsin L proteinases, and those with shorter propeptides lacking this motif, including cathepsin B proteinases[1]. Compared to other papain-like proteinases, the three-dimensional structure of cathepsin B (EC 3.4.22.1) has an extra 20 amino acid peptide segment, termed the occluding loop[2]. This loop partially blocks the end of the active site cleft and positions a positively charged imidazole group of a histidine residue (H111) to accept the negative charge at the C-terminus of the substrate, thus vesting cathepsin B with an exopeptidase activity in addition to its endopeptidase activity[3].

The cDNAs encoding cathepsin B have been isolated from various animals, including vertebrates such as mammals, fish, amphibians and chicken, and invertebrates like nematodes, insects and shrimp. Cathepsin B is expressed in many cells and tissues of several species[4–7]. It has been implicated in a wide range of biological processes, including food digestion[5,7], host tissue digestion[8], metamorphosis[9,10], vitellin degradation[11] and chronic inflammatory diseases[12]. Generally speaking, vertebrate cathepsin B has been well-studied both structurally and functionally, while invertebrate cathepsin B has been less characterized.

Amphioxus or lancelet, a cephalochordate, has long been regarded as the extant invertebrate most closely related to the proximate ancestor of vertebrates. Its genetic information on gene sequence and expression pattern has been widely used for interspecies comparative genome studies and developmental homology analysis. Yet the gene coding for cathepsin B remains unknown in this evolutionarily important animal. The aim of this study was thus to unravel the characteristics and expression profile of cathepsin B gene from amphioxus *Branchiostoma belcheri tsingtauense*.

The gut cDNA library of adult amphioxus was constructed with SMART cDNA Library Constrution Kit (CLONTECH, Palo Alto, CA, USA), according to the method described previously[13]. cDNA clones were selected at random for sequencing. Both strands of all selected clones were sequenced with ABI PRISM 377XL DNA sequencer and all sequences were then analysed for coding probability with the DNATools program. Comparison against the GenBank protein database was performed using the BLAST network server at the National Center for Biotechnology Information (NCBI). Multiple protein sequences were aligned using the MegAlign program by the CLUSTAL method in DNASTAR software package. Phylogenetic tree was constructed by the neighbour-joining method within the PHYLIP 3.5c software package using 1000 bootstrap replicates. Accession numbers of cysteine protease sequences used for comparison were listed in Table 1.

Total RNAs were prepared with Trizol (Gibco) from various tissues, including gill, muscle, testis, ovary, hepatic caecum, hind-gut and notochord of adult amphioxus.

For Northern blotting, a total of 4 μg RNAs each was electrophoresed and blotted onto Nylon membrane (Osmonics Inc.). The blots were hybridized at high stringency with DIG-labelled *AmphiCB* riboprobe of about 1100 bp (1 μg/ml in DIG Easy Hyb) for 15 h at 58°C and the hybridized bands were visualized by BM-Purple (Roche).

For RT–PCR, antisense primer (5′ TGCTCGCTTGGGTTGTCT 3′) and sense primer (5′ CGCAGGGCTTGATCTCGT 3′) were used for synthesis of the amplification product. The reaction was performed according to the in-

Table 1. Representative members of the cysteine protease family

Protein	Abbreviation	Source	Accession number in GenBank	Amino acid
Human cathepsin B	HsCB	*Homo sapiens*	M14221	339
Human cathepsin K	HsCK	*Homo sapiens*	NP_000387	329
Human cathepsin L	HsCL	*Homo sapiens*	AAA66974	333
Human cathepsin H	HsCH	*Homo sapiens*	X16832	335
Human cathepsin W	HsCW	*Homo sapiens*	BC048255	376
Mouse cathepsin B	MmCB	*Mus musculus*	AAH06656	339
Mouse cathepsin K	MmCK	*Mus musculus*	NP_031828	329
Mouse cathepsin L	MmCL	*Mus musculus*	BC051665	330
Mouse cathepsin H	MmCH	*Mus musculus*	BC006878	333
Mouse cathepsin W	MmCW	*Mus musculus*	NP_034115	371
Cow cathepsin B	BoCB	*Bos taurus*	L06075	335
Rat cathepsin B	RnCB	*Rattus norvegicus*	NP_072119	339
Chicken cathepsin B	ChCB	*Gallus gallus*	NP_990702	340
Frog cathepsin B	XeCB	*Xenopus laevis*	BC044689	333
Rainbow trout cathepsin B	RtCB	*Oncorhynchus mykiss*	AAK69705	330
Zebrafish cathepsin B	ZeCB	*Danio rerio*	AAH44517	330
Amphioxus cathepsin B	*AmphiCB*	*B. belcheri tsingtauense*	AAQ83887	333
Shrimp cathepsin B	ShCB	*Pandalus borealis*	AB091671	328
Corn rootworm	CrCB	*Diabrotica virgifera virgifera*	CAE47498	328
Fruit fly	DmCB	*Drosophila melanogaster*	NM_132692	340
Nematode	HcCB	*Haemonchus contortus*	M31112	342

Figure 1. Nucleotide and deduced amino acid sequences of amphioxus *Branchiostoma belcheri tsingtauense* cathepsin B cDNA (*AmphiCB*, accession number in GenBenk: AAQ83887). Antisense primer (5′ TGCTCGCTTGGGTTGTCT 3′) and sense primer (5′ CGCAGGGCTTGATCTCGT 3′) used for RT–PCR are underlined. Down arrows indicate cleavage sites between signal peptide and propeptide, and between propeptide and mature enzyme. Active site cysteine, histidine and asparadine residues were shaded black with white lettering. Two potential N-glycosylation motifs in the amino acid sequence are each put with parenthesis. The polyadenylation signal AATAAA is double underlined. Asterisk represents the stop codon. The numbering of the nucleotide and amino acid sequences is shown to the right.

struction for use of the kit (A1250, Promega), with slight modification. Briefly, after reverse transcription, PCR amplification was carried out in 28 cycles using the following parameters: denaturation at 94°C for 30 s, annealing at 58°C for 1 min and elongation at 68°C for 2 min. Normalization was carried out by amplification of cytoskeletal β-actin mRNA using an antisense primer (5′ GCTGGGCTGTTGAAGGTC 3′) and a sense primer (5′ CTCCGGTATGTGCAAGGC 3′), under the same conditions described above. At the same time, a negative control without RNA template was also run.

The cDNA clone (GenBank accession number: AAQ83887) obtained from the gut cDNA library of amphioxus *B. belcheri tsingtauense* is 1700 bp long, and its longest open

Figure 2. Alignment of cathepsin Bs, including amphioxus *B. belcheri tsingtauense AmphiCB*. Shaded (with solid black) residues are the amino acids that match the consensus. Gaps introduced into sequences to optimize alignment are represented by (-). Signal peptides are indicated by a bracket, residues of the catalytic site by (*) and twelve conserved cysteine residues by (#). Vertical double arrow indicates the cleavage site between propeptide and mature enzyme. The conserved E245 (human numbering) is marked with (+). Conserved occluding loop is boxed. See Table 1 for sequence reference.

reading frame codes for a protein of 333 amino acids with a predicted molecular mass of 36.5 kDa (Figure 1). There is an initiator methionine codon at the 5′ end and a stop codon at the 3′ end. The 3′ untranslated region (UTR) contains an identified polyadenylation signal AATAAA and a polyadenylation tail. Therefore, the cDNA encodes a full-length protein.

Initial BLAST search at NCBI revealed that the protein encoded by the cDNA shared 54% (171/313) and 53% (167/315) identity with zebrafish (NP_957349) and human cathepsin B (AAH10240) respectively. It was therefore compared further with other members of cathepsin B family. Figure 2 shows an alignment of the deduced amino acid sequence with that of cathepsin B proteinases from a representative group of species, including both vertebrates and invertebrates. The protein encoded by the cDNA contains a conserved occluding loop (residues 104–125), a unique feature of cathepsin B. These indicate that the cDNA encodes an amphioxus cathepsin B proteinase, and is thus designated *AmphiCB*.

Resembling other known cathepsin Bs, *AmphiCB* has a putative signal peptide consisting of an N-terminal sequence of 15 amino acids (SignalP 3.0 server), a propeptide containing 64 amino acid residues and a predicted mature enzyme with 254 amino acid residues. The calculated molecular masses for the proprotein and mature enzyme are 34.9 and 27.6 kDa respectively. Similar molecular sizes are found for other known cathepsin B proteinases such as those of shrimp[5], insect[7], rat[14], and mouse and human[15]. Canonical cleavage of the signal peptide is located at Ala^{-65}-Lys^{-64} (Figure 1). The putative cleavage point of the propeptide of the mature enzyme was estimated by homology, to

reside between residues Asp^{-1} and Ile^{1} (Figure 1), fitting the scheme of a conserved proline residue at point 2 in the mature enzyme, as observed in many members of the papain superfamily. This proline may serve to prevent unwanted N-terminal proteolysis[16]. There are also two putative N-linked glycosylation sequences (N-X-S/T) in *AmphiCB* situated in the mature protein. As glycosylation with mannose 6-phosphate has been shown to be an important sorting signal for transporting proteins into lysosomes[3], it thus appears that targeting via mannose 6-phosphate receptor is only possible for mature *AmphiCB*.

In *AmphiCB*, the essential catalytic triad Cys^{29}–His^{198}–Asn^{218} is fully conserved, and the 12 cysteine residues that form six disulphide bonds[11] are also present (Figure. 2). Moreover, *AmphiCB* has two conserved His residues (His^{109} and His^{110}) in the occluding loop, which are thought to be responsible for the exopeptidase activity in mammalian cathepsin B[2]. It has been known that the presence of the residue Glu at position 245 in human cathepsin B is essential for its activity to hydrolyse the substrate Z-Arg-Arg-MCA because its replacement with Ala was found to cause the loss of this activity[17]. It is of interest to note that this residue was substituted with Asp in *AmphiCB* (position 244) and in shrimp cathepsin B (position 245)[5]. Whether the substitution impairs the hydrolysis specificity of these proteinases remains to be determined.

To shed light on the evolutionary position of *AmphiCB*, an unrooted phylogenetic tree was constructed using the amino acid sequence of *AmphiCB* and those of different cysteine proteinases. It revealed the existence of two main clades, one consisting of cathepsin B and the other comprising cathepsin L, cathepsin K, cathepsin H and cathepsin W. As expected, *AmphiCB* is clustered with cathepsin B clade in the tree (Figure 3). Evolutionarily, *AmphiCB* appears more closely related to invertebrate cathepsin B.

Northern blotting was conducted to assess the size of the transcript and its tissue distribution. As shown in Figure 4 *a*, a 1700 bp band of *AmphiCB* transcript was detected in the hind-gut and hepatic caecum, and although at much lower level, it was also present in the testis and muscle. In contrast, hybridization signals were not observed in the

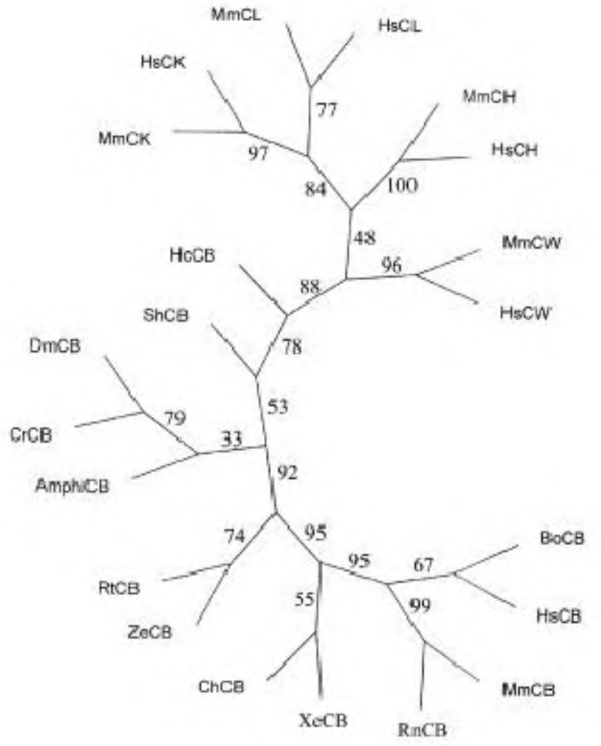

Figure 3. Unrooted phylogenetic tree of *AmphiCB* and various cysteine proteases constructed by neighbour-joining method within the package PHYLIP 3.5c. Bootstrap majority consensus values on 1000 replicates are indicated at each branch point in per cent. See Table 1 for sequence reference.

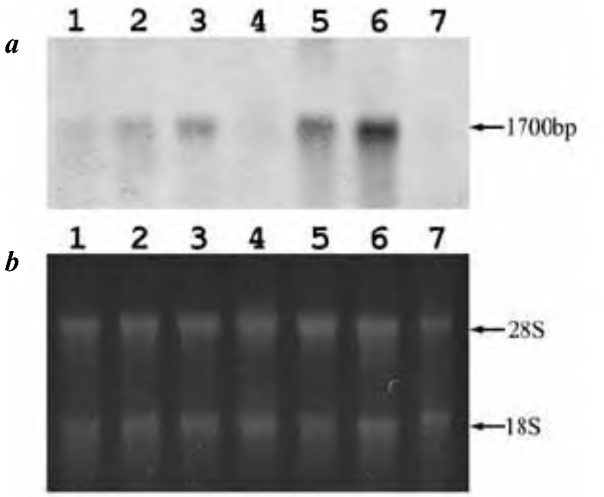

Figure 4. Northern analysis for *AmphiCB* transcripts in different tissues of amphioxus. Lane 1, Gill; lane 2, Muscle; lane 3, Testis; lane 4, Ovary; lane 5, Hepatic caecum; lane 6, Hind-gut; lane 7, Notochord. ***a***, The blot was hybridized with dig-labelled amphioxus *AmphiCB* RNA probe. Arrow indicates the position of molecular size equivalent to 1700 bp. ***b***, A total of 4µg RNA for each sample was analysed in 1.2% agarose formaldehyde-denaturing gel.

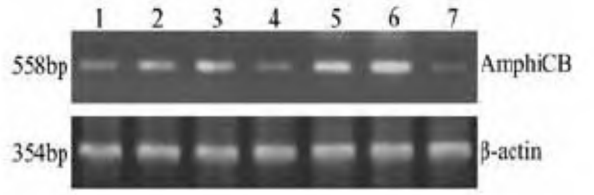

Figure 5. RT–PCR amplification of *AmphiCB* mRNAs from different tissues of amphioxus. A band of 558 bp is observed in all tissues examined. RT–PCR of amphioxus cytoskeletal β-actin mRNA was used for normalization. Lane 1, Gill; lane 2, Muscle; lane 3, Testis; lane 4, Ovary; lane 5, Hepatic caecum; lane 6, Hind-gut; lane 7, Notochord.

gill, ovary and notochord. To further confirm tissue distribution, the more sensitive RT–PCR technique was performed. An amplification product of the expected size (558 bp; see Figure 1 for primer position) was observed in all tissues examined, but signals for gill, ovary and notochord were comparatively weak (Figure 5). The relative abundance of *AmphiCB* transcript in the hind-gut and hepatic caecum suggests a possible digestive role of *AmphiCB* in the amphioxus alimentary canal. However, the ubiquitous expression of *AmphiCB* in amphioxus implies it may also play a housekeeping role. This merits further study at the protein level.

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Insecticide susceptibility status of malaria vectors in some hyperendemic tribal districts of Orissa

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Insecticide susceptibility status of adult, wild-caught *Anopheles culicifacies* and *An. fluviatilis* against diagnostic dosages of DDT (4%), malathion (5%) and deltamethrin (0.05%) was determined according to standard WHO procedure in some districts of Orissa. All these districts are predominantly inhabited by the tribal population and are hyperendemic for malaria. The results showed that *An. culicifacies* is resistant to DDT in all the eight districts, to malathion in Mayurbhanj, Bolangir, Nuapada and Kalahandi districts and is showing signs of development of multiple resistance to DDT, malathion and deltamethrin in Bolangir, Nuapada and Kalahandi districts. *An. fluviatilis* was found susceptible to DDT, malathion and deltamethrin in all the districts except Mayurbhanj, where 95 and 87.5% mortality was observed against DDT and malathion respectively. The delayed knock-down effect of deltamethrin in *An. culicifacies* (KDT_{50}: 11.78–25.31 min; KDT_{90}: 24.20–65.22 min) and *An. fluviatilis* (KDT_{50}: 20.87–25.19 min; KDT_{90}: 45.81–54.11 min) was observed in all the districts, which is an indication of incipient resistance. Based on these findings, appropriate changes in the indoor residual spray strategy have been suggested to achieve effective vector control.

VECTOR control programmes in India rely mostly on indoor residual spraying by DDT[1]. The spectacular success achieved

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