

## Alanine Aminotransferase in Amphioxus: Presence, Localization and Up-regulation after Acute Lipopolysaccharide Exposure

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Received 14 February 2006, Accepted 13 April 2006

Alanine aminotransferase (AAT) is mainly synthesized in the liver, and its level in mammalian serum is elevated after acute phase induction. Here we demonstrated that sheep anti-human AAT antibody cross-reacted with amphioxus humoral fluids as well as human serum; and the concentration of AAT in the humoral fluids in amphioxus increased after the acute challenge with lipopolysaccharide, while the level of total proteins remains unchanged. These suggest the presence of the same acute phase response pattern in amphioxus, as observed in some mammalian species. Immunohistochemically, AAT was localized in the hepatic diverticulum, ovary and testis. It appears that the hepatic diverticulum in amphioxus is functionally homologous to the vertebrate liver in respect of AAT synthesis, supporting the hypothesis that the vertebrate liver evolved from the hepatic diverticulum of an amphioxus-like ancestor during early chordate evolution.

**Keywords:** Acute phase response, Alanine aminotransferase, Amphioxus, *Branchiostoma*, Diverticulum

### Introduction

Alanine aminotransferase (AAT; EC 2.6.1.2.), formerly known as glutamate pyruvate transaminase (GPT), is a pyridoxal enzyme, which catalyzes reversible transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate. AAT is primarily synthesized in the liver (Segal *et al.*, 1962; Gatehouse *et al.*, 1967; Walton and Cowey, 1982; Alexin and Papoutsoglou, 1986; Gubern *et al.*, 1990; Ishiguro *et al.*, 1991; Matsuzawa *et al.*, 1997) though its activities exist in other tissues including heart, muscle, kidney and brain

(Milton *et al.*, 1967; McKenna *et al.*, 1996). AAT is an index for diagnosis of liver function, and it has been shown that serum AAT activity is significantly elevated during liver damage caused by drug toxicity and infection while the total protein level remains constant (Patnaik and Kanungo, 1976; Sherman, 1991; Agarawal *et al.*, 1996; Cahen *et al.*, 1996; Rodriguez *et al.*, 1997; Dufour *et al.*, 2000).

AAT has been documented in various species ranging from fungi to mammals (Gatehouse *et al.*, 1967; Chen, 1985; Umemura *et al.*, 1994; Zelada *et al.*, 1996; Yang *et al.*, 2002; Chien *et al.*, 2003; Treberg *et al.*, 2003; Srivastava *et al.*, 2004). Extensive studies have been done on the metabolic role of this enzyme in a number of mammals including human (Felig, 1973; Derosa and Swick, 1975; Peng *et al.*, 1991). However, information on AAT in invertebrates is restricted only to few species such as fruit-fly (Chen, 1985), shrimp (Chien *et al.*, 2003) and lobster (Devereaux, 1986).

Amphioxus (protochordate) has been regarded as an organism most closely related to the ancestor of ancient vertebrates (Ruppert, 1997; Holland *et al.*, 2004) since the first description of its embryogenesis by Kowalevsky in 1877. It has a circulation system with the fundamental organization of all chordates (Rähr, 1979). Our previous investigation has shown that the humoral fluid including the blood in amphioxus contains phenoloxidase, lectin and complement component C3 (Wang *et al.*, 2002; Zhang *et al.*, 2003; Pang *et al.*, 2004a; 2005b), but little is known concerning the plasma proteins like AAT in amphioxus to date. The aims of this study were thus to examine if the humoral fluid in amphioxus has AAT, and if so, to determine its localization, and to detect if its production shares characteristics with the homologues in mammalian species.

### Material and Methods

**Antibodies and chemicals.** Sheep anti-human AAT antibody was purchased from US Biological (USA), and peroxidase-conjugated

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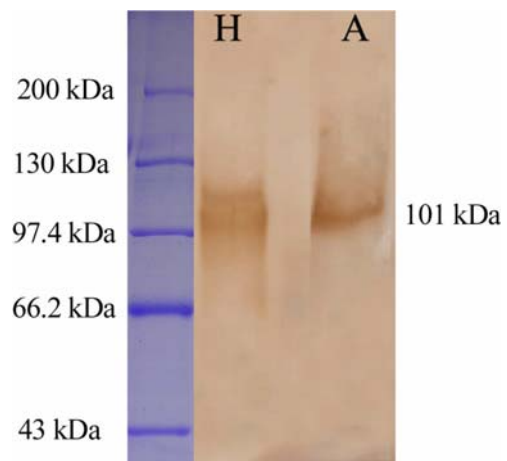
donkey anti-sheep IgG was from Jackson (USA). Lipopolysaccharide (LPS), and 3,3'-diaminobenzidine (DAB) were both from Sigma (USA). All other chemicals used were analytical reagents.

**Preparation of humoral fluid.** Amphioxus *Branchiostoma belcheri* with average body length of about 4 cm were collected from the "amphioxus ground" near Shazikou in the vicinity of Qingdao. A total of 2000 amphioxus were cultured in 1 liter of filtered natural seawater containing 10 µg/ml LPS. At different intervals (12, 24, 48 and 72 hr) after treatment, 300 of amphioxus *B. belcheri* were sampled, and rinsed with distilled water. The humoral fluids were prepared according to the method of Wang *et al.* (2002). Briefly, the rinsed amphioxus were wiped out thoroughly with sterilized gauze, cut into about 2 mm<sup>3</sup> pieces on ice to bleed, and centrifuged at 12,000 × *g* for 30 min at 4°C. The supernatants were pooled and stored at -70°C until used. The humoral fluid was also prepared similarly from untreated amphioxus.

**SDS-PAGE and Western blotting.** The humoral fluid prepared from normal amphioxus was run on a 7% SDS-PAGE gel with a 4% spacer gel using the buffer system of Laemmili (1970). Human serum was also run on the gel as positive control at the same time. The gels were washed for 5 min in the transfer buffer of 15.6 mM Tris-HCl containing 120 mM glycine and 20% methanol (pH 8.3), and proteins on the gels were blotted on nitrocellulose membrane (Hybond, Amersham Pharmacia). Blotted membranes were incubated in 20 mM phosphate-buffered solution (PBS; pH 7.4) containing 50 mM NaCl and 5% defatted milk powder at room temperature for 1 hr, washed three times with 20 mM PBS containing 50 mM NaCl, and then cross-reacted with sheep anti-human AAT antibody diluted 1 : 800 with 20 mM PBS (pH 7.4) containing 50 mM NaCl and 5% defatted milk powder at room temperature for 1.5 hr. After washing in 20 mM PBS, the membranes were incubated with peroxidase-conjugated donkey anti-sheep IgG diluted 1 : 1000 with PBS at room temperature for 45 min. Bands were visualized using 0.06% DAB in 50 mM Tris-HCl buffer (pH 7.6) and 0.03% H<sub>2</sub>O<sub>2</sub>. The molecular mass standards used were rabbit actin (43 kDa), bovine serum albumin (66.2 kDa), rabbit phosphorylase b (97.4 kDa), calmodulin binding protein (130 kDa) and myosin (200 kDa).

**Determination of total proteins and AAT in humoral fluid.** The levels of total proteins and AAT in the humoral fluids from normal and LPS-treated amphioxus were all determined using Abbott diagnostics test kits (Abbott, USA) by an AEROSET automatic biochemical analyzer (Abbott, USA).

**Immunohistochemistry.** Amphioxus *B. belcheri* were each severed into 3 to 4 pieces, and fixed in freshly prepared 4% (w/v) paraformaldehyde in 100 mM PBS (pH 7.4) at 4°C for 24 hr. After dehydration, they were embedded in paraffin, and sectioned at 5 µm. The sections were mounted on slides, and dried at 42°C for 4 hr. They were dewaxed in xylene for 10 min (two changes for 5 min each) followed by immersion in absolute ethanol for 10 min (two changes for 5 min each), and then re-hydrated in 95%, 90%, 80% and 70% ethanol (one change for 5 min) and brought to 100 mM PBS. After rinsing with distilled water for 5 min, the endogenous peroxidase activity in the sections was quenched with incubation in 3% (v/v) H<sub>2</sub>O<sub>2</sub> at room temperature for 15 min, which was



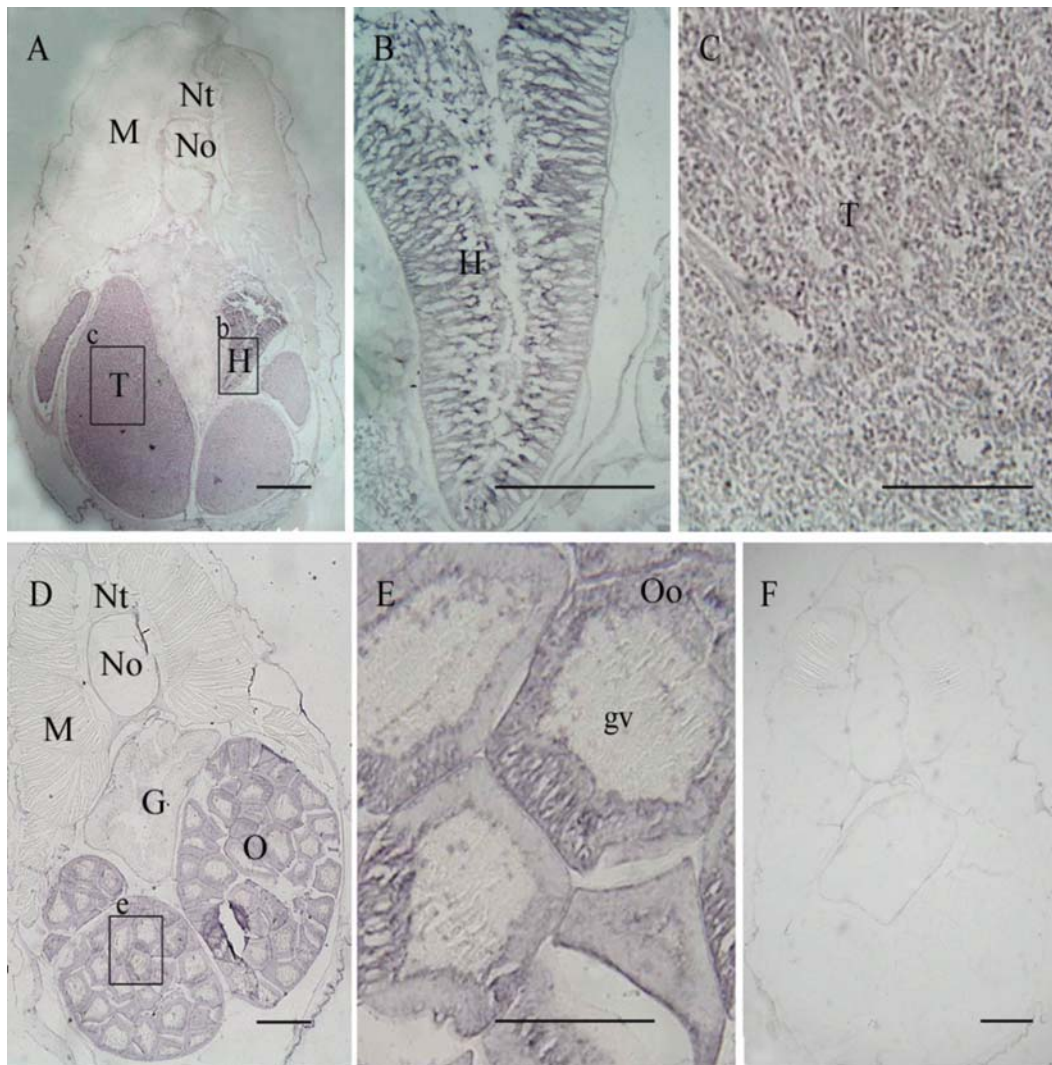
**Fig. 1.** Western blotting analyses of human and amphioxus AAT. Sheep anti-human AAT antibody cross-reacted with amphioxus humoral fluids (lane A) as well as human serum (lane H). The human serum was reactive with sheep anti-human AAT antibody, producing two close bands representing the isozymes AAT1 and AAT2, and the amphioxus humoral fluids reacted with sheep anti-human AAT antibody, forming a single positive band with an apparent molecular mass of approximately 101 kDa corresponding to the molecular weight of human AAT1.

followed by 5 min wash in redistilled water. Subsequently, the sections were pre-incubated with 5% defatted milk powder in 20 mM PBS (pH 7.4) at room temperature for 30 min, washed in 20 mM PBS for 5 min, and then incubated overnight with sheep anti-human AAT antibody diluted 1 : 500 with 20 mM PBS containing 3% defatted milk powder in a humidified chamber at 4°C. The control sections were similarly incubated with pre-immune sheep serum. Both experimental and control sections were washed three times for 3 min each in 20 mM PBS, and incubated further with peroxidase-conjugated donkey anti-sheep IgG diluted 1 : 1000 with PBS at room temperature for 1 hr. The chromogenic reaction was achieved by addition of 0.015% (w/v) DAB containing 0.1% NiCl<sub>2</sub> and 0.02% (v/v) H<sub>2</sub>O<sub>2</sub> in 50 mM Tris-HCl buffer (pH 7.6) and maintaining in the dark for 5 min. The sections were mounted in Canada balsam, observed and photographed under a BX51 Olympus microscope.

## Results

Western blotting analysis showed that sheep anti-human AAT antibody cross-reacted with amphioxus humoral fluids as well as human serum. The human serum was reactive with sheep anti-human AAT antibody, producing two close bands representing the isozymes AAT1 and AAT2 (Jadaho *et al.*, 2004), respectively. In contrast, the amphioxus humoral fluids reacted with sheep anti-human AAT antibody, forming a single positive band with an apparent molecular mass of approximately 101 kDa corresponding to the molecular weight of human AAT1 (Fig. 1).

The immunohistochemical staining demonstrated that AAT



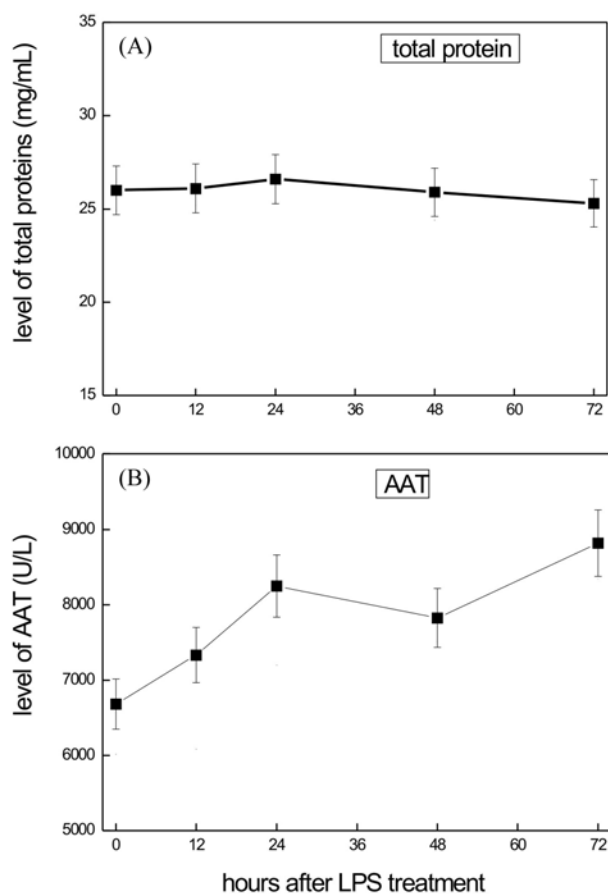
**Fig. 2.** Immunohistochemical localization of amphioxus AAT in different tissues. AAT was detected in the hepatic diverticulum, testis and ovary (A, B, C, D and E), and no staining was seen in the control sections treated with pre-immune sheep serum (F). B and C are magnifications of the boxes b (hepatic diverticulum) and c (testis) in A, and E is magnification of box e (oocyte) in D. H, hepatic diverticulum; M, myotomes; No, notochord; Nt, neural tube; G, gut; T, testis; O, Ovary; Oo, Oocyte; gv, germinal vesicle. Bars represent 100  $\mu$ m.

was detected in the hepatic diverticulum, testis and ovary, while it was not detectable in other tissues such as notochord, muscle and hind gut (Fig. 2A, B, C, D and E). The positive signals for AAT were observed in the cytoplasm of the diverticulum cells, oocyte and spermatozoa. In contrast, no staining was seen in the control sections treated with pre-immune sheep serum (Fig. 2F)

Figure 3 shows the changes in the concentrations of total proteins and AAT in the humoral fluids at different times after acute LPS treatment. The content of total proteins in the humoral fluids remained basically unchanged (Fig. 3A). In contrast, the concentration of AAT increased to about 110% at 12 hr after treatment, and then to 131, 117 and 132% after 24, 48 and 72 hr, respectively (Fig. 3B).

## Discussion

Here we demonstrated for the first time that AAT is present in the humoral fluids in amphioxus *B. belcheri* and AAT is immunohistochemically localized in the hepatic diverticulum. It is of interest to note that human AAT consists of two close bands, which may represent AAT1 and AAT2 isoenzymes as found in mouse (Jadaho, 2004), while only one form of AAT is present in amphioxus. The primary synthesis site of AAT in the vertebrates is the liver, which is an endoderm-derived organ and unique to all vertebrates. Amphioxus has a hepatic diverticulum, 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



**Fig. 3.** Changes in the levels of total proteins and AAT in the humoral fluids at different times (0, 12, 24, 48, 72 hr) after acute LPS challenge (10 µg/ml LPS). The content of total proteins in the humoral fluids remained basically unchanged (A,  $n=3$ ), while the concentration of AAT increased to about 110% at 12 hr after treatment, and then to 131, 117 and 132% after 24, 48 and 72 hr, respectively (B,  $n=3$ ).

considered to be the precursor of vertebrate liver (Müller, 1844; Hammar, 1898; Welsch, 1975; Ruppert, 1997). In respect of AAT synthesis the hepatic diverticulum in amphioxus is apparently equivalent to the vertebrate liver. This provides an evidence of physiological function supporting the hypothesis that the vertebrate liver evolved from the hepatic diverticulum of an amphioxus-like ancestor initially proposed by Müller (1844). It is of interest to note the presence of AAT in ovary and testis. The origin of AAT in the two tissues is unknown at present. One possibility is that AAT, like vitellogenin (Wallace, 1985; Sun and Zhang, 2001), is synthesized in the hepatic diverticulum secreted into the blood stream, transported to the gonads where it is taken in by the developing gametes. Further studies on AAT gene transcription will shed light on this problem.

It has been shown that AAT level in serum of rats is significantly increased after LPS challenge (Kim and Kim, 2002). The concentration of AAT in the humoral fluids in

amphioxus is also markedly elevated following LPS treatment, while the total protein level in the fluids remains constant. Like what has been found in rats (Kim and Kim, 2002), the elevation of AAT in amphioxus humoral fluids may be due to the damage of the hepatic diverticulum cells caused by LPS, which results in the leakage of AAT into the humoral fluid. Moreover, the fluctuation pattern of AAT level in amphioxus humoral fluids is also similar to that observed in rats after acute phase response, suggesting that a mammalian-like acute phase response system may be present in the protochordate *B. belcheri*.

**Acknowledgments** This work was supported by the Natural Science Foundation of China (NSFC; 30470203) and the Ministry of Science and Technology (MOST) of China.

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