# Presence and Characterization of Complement-like Activity in the Amphioxus Branchiostoma belcheri tsingtauense

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# Presence and Characterization of Complement-like Activity in the Amphioxus *Branchiostoma belcheri tsingtauense*

Shicui Zhang[1]*, Changfa Wang[1], Yongjun Wang[1], Ran Wei[1], Guohu Jiang[2] and Hong Ju[1]

[1]*Department of Marine Biology, Ocean University of China Qingdao 266003, PR China*
[2]*No 2 People's Hospital of Qingdao, Qingdao 266003, PR China*

**ABSTRACT**—The humoral fluid of *Branchiostoma belcheri tsingtauense* was examined for the presence of complement-like activity. The humoral fluid showed hemolytic activity for rabbit erythrocytes and those from species representing mammals, birds, amphibians and fish, but not sensitized sheep erythrocytes. There was no relationship between phylogeny of the target erythrocytes and degree of hemolysis. The hemolytic activity was optimally assayed at 20°C, at pH 7.5, and in the presence of 10 mM $Mg^{2+}$. The hemolytic activity was $Mg^{2+}$-dependent and heat-sensitive, and was abrogated by treatment with rabbit anti-human C3 serum, zymosan, methylamine, hydrazine, and phenylmethylenesulfonyl fluoride. In addition, Western blotting and titration by turbidimetric immunoassay (TIA) revealed that amphioxus humoral fluid contained C3 component, and its concentration is about 1.17 mg/ml, which is comparable to C3 concentration in human or dog sera. These suggest that the hemolytic activities displayed by amphioxus humoral fluid appear to represent the vertebrate complement system probably operating via the alternative pathway.

**Key words:** amphioxus, humoral fluid, hemolytic activity, alternative pathway (AP), complement 3 (C3)

## INTRODUCTION

Complement is a complex system of serum proteins that plays a pivotal role in vertebrate defense reactions. Activation of the mammalian complement system occurs via three convergent pathways, the classical pathway (CP) which is activated by an antigen-antibody complex and requires $Ca^{2+}$ and $Mg^{2+}$ (Ruddy, 1974), the alternative pathway (AP) which is triggered by the third component of complement C3 and requires $Mg^{2+}$ alone, and the lectin pathway which is activated by mannose-binding lectin (MBL) that interacts with mannose sugars on bacterial cell surfaces (Smith *et al*., 1999). In contrast to the CP, the AP activity is susceptible to treatment with the mammalian complement inactivators like zymosan (which is a C3-depleting agent), hydrazine (which destroys C3 and C4 components of mammalian complement), and methylamine (which is commonly used in complement analysis to deactivate the thioester sites on complement proteins) (Takada *et al*., 1978; Gotze and Müller-Eberhard, 1976; Vogel and Müller-Eberhard, 1985; Van Dijk *et al*., 1980; Suner and Tort, 1994). Phylogenetically, the relation of the AP and the lectin pathway remains open (Al-Sharif *et al*., 1998; Zarkadis *et al*., 2001; Smith *et al*., 1998), yet the CP may have generated from the alternative and lectin pathways by a genome wide duplication during early period of vertebrate evolution (Ohno, 1970; Zarkadis *et al*., 2001).

The CP activity can be assayed by determining the amount of serum required to hemolyse a given number of sensitized sheep red blood cells (SRBC), whereas the AP activity is measured in the same manner, but by using rabbit red blood cells (RaRBC) instead of sensitized SRBC. It has been demonstrated that functionally and structurally similar complement components are present in all non-mammalian vertebrates such as birds (Ohta *et al*., 1983; Kai *et al*., 1983), reptiles (Vogel *et al*., 1984; Koppenheffer, 1987), amphibians (Sekizawa *et al*., 1984) and fish including hagfish and lamprey, the most primitive class of extant vertebrates (Koppenheffer, 1987; Hanley *et al*., 1992; Nonaka *et al*., 1984a, b). It has also been shown that complement components are present in invertebrates such as tunicate and sea urchin (Nonaka *et al*., 1999; Al-Sharif *et al*., 1998; Smith *et al*., 1999).

Amphioxus, a cephalochordate, has long been regarded as a basal lineage of chordates. It lacks free circulating blood cells in the circulation system (Moller and Philpott, 1973; Silva *et al*., 1995), and thus study of its immune

* Corresponding author: Tel. +86-532-2032787;
FAX. +86-532-2032276.
E-mail: zscshab@public.qd.sd.cn

defense system would be unexceptionally intriguing. Previous investigation has showed that both lectin and phenoloxidase are involved in defense reactions of amphioxus (Benedicts and Capalbo, 1981; Li *et al*., 2000; Mock and Renwrantz, 1987; Wang *et al*., 2002; Zhang and Li, 2000). However, little is known about the complement system of this animal although from it C3-like and C6-like gene fragments have been cloned (Suzuki *et al*., 2002).

In this study, we have sought to determine whether the humoral fluid of the amphioxus *Branchiostoma belcheri tsingtauense* possesses hemolytic activity, and if so, to detect whether it shares characteristics with the vertebrate complement system. It is reported here for the first time that a hemolytic activity is present in amphioxus humoral fluid, which appears to represent the vertebrate complement system probably operating via the AP.

## MATERIALS AND METHODS

### Reagents

Ethylenediamine tetraacetic acid (EDTA), ethyleneglycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), methylamine hydrochloride, hydrazine hydrate, zymosan, and phenylmethylenesulfonyl fluoride (PMSF) were purchased from Sigma (St Louis, MO), and gelatin and glucose from Amersco (Solon, OH). All the other chemicals used were analytical reagents.

Rabbit anti-human C3 serum (3.44 mg/ml; titer, 1:32) and non-immune rabbit serum were purchased from Sino-American Biotechnology Co. (Beijing), the turbidimetric immunoassay (TIA) kits containing sheep anti-human C3 sera (86 μg/ml) and non-immune sheep sera from Bio-Sun Co. (Fuzhou, Fujian).

The buffers used in the experiments for AP activity assay were 10 mM EGTA-Mg-GVB (pH 7.5) containing 10 mM EGTA, 10 mM $Mg^{2+}$ and GVB (5 mM sodium barbiturate, 446 mM NaCl and 0.1% gelatin), and 10 mM EDTA-GVB (pH 7.5) containing 10 mM EDTA, 5 mM sodium barbiturate, 446 mM NaCl and 0.1% gelatin. Unless otherwise noted, 10 mM EGTA-Mg-GVB was employed. The buffers used in the experiments for CP activity assay were $GVB^{2+}$ containing 5 mM sodium barbiturate, 0.5 mM $Ca^{2+}$, 1.0 mM $Mg^{2+}$, 446 mM NaCl and 0.1% gelatin, and $GGVB^{2+}$ containing 5 mM sodium barbiturate, 0.5 mM $Ca^{2+}$, 1.0 mM $Mg^{2+}$, 446 mM NaCl, 2.5% glucose and 0.1% gelatin. Unless otherwise noted, $GVB^{2+}$ was employed. All buffered solutions were stored at 4°C and used within one week.

### Preparation of amphioxus humoral fluid

Amphioxus *B. belcheri tsingtauense* collected from the "amphioxus ground" near Shazikou in the vicinity of Qingdao were reared in laboratory and fed on a diet of single-cell algae daily until use. The seawater was aerated continuously and changed once a day. The humoral fluid was prepared by the method of Wang *et al*. (2002). Briefly, about one thousand of amphioxus with average of body length of about 4 cm were rinsed with distilled water, wiped out thoroughly with sterilized gauze, and then cut into about 2 $mm^3$ pieces on ice to bleed. After centrifugation at 12 000 g for 30 min at 4°C, the supernatant was collected and stored at –70°C until used.

The protein concentration was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as standard.

### Target erythrocytes

For most experiments target erythrocytes were drawn from the same giant New Zealand male rabbit and prepared for use by washing 3 times with 10 mM EGTA-Mg-GVB with 149 mM NaCl. Erythrocytes were also collected from representative species of major vertebrate classes including human, sheep, rat, chick, pigeon, toad (*Bufo gargarizans*), grass carp (*Ctenopharyngodon idellus*), loach (*Misgurnus anguillicaudatus*) and lip shark (*Chiloscyllium plagiosum*). All blood samples were each mixed with an equal volume of Alsever's solution immediately after sampling and centrifuged at 500 g for 5 min. The pelleted erythrocytes were washed 3 times with 10 mM EGTA-Mg-GVB with 149 mM NaCl and adjusted spectrophotometrically at 414 nm to a concentration of $2\times10^8$ cells/ml, and stored at 4°C.

### Alternative complement pathway titer (ACH50) assay

ACH50 assay was performed according to the method of Yano (1992). Pilot experiments showed that dilution of amphioxus humoral fluid resulted in decrease in its hemolytic activity, and when it was diluted 100-fold with 10 mM EGTA-Mg-GVB, the hemolytic activity was no longer detectable. Therefore, 50, 100, 150, 200 and 250 μl of the humoral fluid diluted 20-fold were dispensed into 5 ml test tubes, respectively, and the total volume was made up to 350 μl with 10 mM EGTA-Mg-GVB. To each test tube was 100 μl of RaRBC suspension ($2\times10^8$ cells/ml) added. A 100% hemolysis control and a cell blank control were included. The tubes were incubated at 20°C for 90 min with frequent shaking and the hemolytic reaction was then terminated by adding cold 3.15 ml 10 mM EDTA-GVB (pH7.5). After centrifugation at 1500 g for 5 min, absorbance of the supernatants at 414 nm ($OD_{414}$) was measured spectrophotometrically. The absorbances including 100% hemolysis control were corrected by subtraction of the absorbance of the cell blank. The ACH50 (units/ml) was calculated according to the method described by Yano (1992).

In addition to RaRBC, the remaining erythrocytes collected were similarly tested to determine the best activators of the AP in the amphioxus.

### Classical complement pathway titer (CH50) assay

The CP activity of amphioxus humoral fluid, if any, was assayed by the method of Yano (1992). SRBC were washed three times with 10 mM PBS (pH7.4), and adult lip sharks (750 g each) were injected peritoneally with 1 ml of the erythrocyte suspension ($5\times10^8$ cells/ml). The fish were successively injected five times at a weekly interval, and bled one week after the final booster injection. For preparation of sensitized SRBC, lip shark antiserum was first heated at 50°C for 30 min to inactivate endogenous complement, diluted 50-fold with 10 mM EDTA-GVB, and mixed with an equal volume of SRBC suspension ($1\times10^9$ cells/ml EDTA-GVB) with constant swirling. The mixture was incubated at 25°C for 30 min, and the sensitized cells were then washed three times with $GGVB^{2+}$. After centrifugation at 500 g, the sensitized SRBC was re-suspended in $GGVB^{2+}$ and adjusted to a concentration of $5\times10^8$ cells/ml.

To examine CH50 of amphioxus humoral fluid, lip shark normal serum, grass carp normal serum and amphioxus humoral fluid were diluted with $GVB^{2+}$ at ratios of 1:50, 1:10 and 1:20, respectively. Different volumes of the diluted sera and amphioxus humoral fluid ranging from 0.4 to 1.0 ml were dispensed into test tubes and the total volume was made up to 1.3 ml with $GVB^{2+}$. To each tube was 0.2 ml of sensitized SRBC suspension ($5\times10^8$ cells/ml) added. A 100% hemolysis control and a cell blank control were included. The tubes were incubated at 25°C for 60 min with occasional shaking, and centrifuged at 1000g for 5 min. Absorbance of the supernatants at 541 nm ($OD_{541}$) was measured spectrophotometrically. The $OD_{541}$ values including 100% hemolysis control were corrected by subtraction of the absorbance of the cell blank. The calculation of CH50 (units/ml) was according to the method of Yano (1992).

### Divalent cation analysis

Pilot experiments showed that addition of 50 μl of 100 mM

EDTA (pH 8.5) or 100 mM EGTA (pH 7.5) into 50 μl of amphioxus humoral fluid had little effect on pH value of the humoral fluid. For divalent cation requirement assay, 50 μl of amphioxus humoral fluid was mixed with 50 μl of 100 mM EDTA (pH 8.5) or EGTA (pH 7.5), and incubated at 25°C for 30 min. The mixtures were then diluted 10-fold with 10 mM EGTA-Mg-GVB, and their hemolytic activity was examined, and ACH50 calculated. Meanwhile, 50 μl of amphioxus humoral fluid was mixed with 50 μl of 100 mM EDTA and pre-incubated at 25°C for 30 min. The mixtures were extensively dialyzed against 10 mM EGTA-GVB for 2 hr at 4°C to eliminate EDTA, and mixed again with 100 μl of 80 mM $CaCl_2$ or $MgCl_2$, and their hemolytic activity was similarly examined and ACH50 calculated.

**Inactivator assay**

A total of 50 μl of amphioxus humoral fluid was mixed with an equal volume of 60 mg/ml zymosan (Day *et al*., 1970). For control, amphioxus humoral fluid was mixed with 10 mM EGTA-Mg-GVB. The mixtures were incubated at 25°C for 30 min, and centrifuged at 12 000 g for 10 min. The supernatants were pooled, diluted 10-fold with 10 mM EGTA-Mg-GVB, and their hemolytic activity was determined, and ACH50 calculated.

To test the effects of methylamine and hydrazine on the hemolytic activity, 50 μl of amphioxus humoral fluid was mixed with an equal volume of 6.3, 12.5, 25, 50, 100 and 200 mM methylamine or hydrazine prepared in 10 mM EGTA-Mg-GVB (Vogel and Müller-Eberhard, 1985). For control, amphioxus humoral fluid was mixed with 10 mM EGTA-Mg-GVB. The mixtures were pre-incubated at 25°C for 30 min, and diluted 10-fold with 10 mM EGTA-Mg-GVB. After incubation for 90 min at 20°C, their hemolytic activity was determined, and ACH50 calculated.

Similarly, 50 μl of amphioxus humoral fluid was mixed with an equal volume of 1, 2, 5, 10 and 20 mM PMSF prepared in 2-propanol (Leonard *et al*., 1990). For control, amphioxus humoral fluid was mixed with 2-propanol. The mixtures were pre-incubated at 25°C for 30 min, and diluted 10-fold with 10 mM EGTA-Mg-GVB. After incubation for 90 min at 20°C, their hemolytic activity was determined, and ACH50 calculated.

**Anti-human C3 serum treatment assay**

The capacity of the rabbit anti-human C3 serum to inhibit the hemolytic activity of amphioxus humoral fluid was analyzed by the method of Nonaka *et al*. (1999). The rabbit antiserum was inactivated at 56°C for 20 min, and diluted two-fold serially up to 32 times with 10 mM PBS (pH7.4) containing 446 mM NaCl. Aliquots of 50 μl of the serially diluted antiserum were mixed with an equal volume of amphioxus humoral fluid, and the mixtures were incubated at 25°C for 1 hr. After centrifugation at 3000 g for 10 min, the supernatants were diluted 10-fold with 10 mM EGTA-Mg-GVB, and their hemolytic activity was determined. For control, both heat-inactivated non-immune rabbit serum and 10 mM PBS (pH7.4) containing 446 mM NaCl were incubated with amphioxus humoral fluid.

To confirm the presence of C3 component in amphioxus humoral fluid, Western blotting analysis was carried out. Amphioxus humoral fluid and human serum were electrophoresed on 7.5% native PAGE (Davis, 1964). Proteins separated were transferred onto nitrocellulose membrane and immunostained with rabbit anti-human C3 serum (Towbin *et al*., 1979).

**Titration of C3 protein**

The protein concentration of amphioxus humoral fluids prepared was about 14.46 mg/ml. The content of C3 component in amphioxus humoral fluid was assayed according to the instructions of the TIA kits. For control, the concentration of C3 in 5 healthy human sera was also assayed. An aliquot of 250 μl of sheep anti-human C3 serum (86 μg/ml) was mixed with 25 μl of amphioxus humoral fluid diluted 5-fold with 10 mM PBS (pH7.4), and the mixture was incubated at 25°C for 200 seconds, and absorbance measured by an automated chemistry analyzer AU 560 (Japan), which had been pre-adjusted to zero with non-immune sheep serum containing in TIA kits. The C3 concentration in human sera was similarly measured.

**Optimization of ACH50 assay**

To examine the optimum temperature for the hemolytic reaction, seven groups of samples including different volumes of the diluted humoral fluid and 100 μl of RaRBC suspension ($2\times10^8$ cells/ml) prepared as described above in "Alternative complement pathway titer (ACH50) assay" were incubated at 4°C, 20°C, 25°C, 37°C, 45°C, 50°C and 56°C, respectively, for 90 min (Matsuyama *et al*., 1988). The hemolytic reaction was terminated by adding a volume of 3.15 ml cold EDTA-GVB (10 mM, pH7.5), and the hemolytic activity then measured and ACH50 calculated.

To test the optimum pH, the buffer 10 mM EGTA-Mg-GVB was readjusted to pH 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0, respectively, and used in the hemolytic activity analysis (Matsuyama *et al*., 1988). The hemolytic reactions were carried out at 20°C for 90 min, and ACH50 was calculated from the $OD_{414}$.

To assay the $Mg^{2+}$ optimum concentration, the concentration of $Mg^{2+}$ in the buffer 10 mM EGTA-GVB was adjusted to 0, 5, 10 and 15 mM, respectively, and used in the hemolytic activity analysis (Matsuyama *et al*., 1988). The hemolytic reactions were carried out at 20°C for 90 min, and ACH50 was calculated.

## RESULTS

### Hemolytic activity

The hemolytic reaction was first tested in the presence of 10 mM EGTA, which was sufficient to prevent any CP activation due to natural antigen-antibody complexes, if any. The hemolytic assay revealed that amphioxus humoral fluid was capable of hemolysing RaRBC suspended in EGTA-Mg-GVB (Table 1). In contrast, the humoral fluid failed hemolysing sensitized SRBC suspended in $GVB^{2+}$ though the sensitized SRBC had been proved to be excellent acti-

**Table 1.** Sensitivity of different erythrocyte species to hemolysis by amphioxus humoral fluid.

| Erythrocyte species | ACH50 (units/ml) |
|---|---|
| Human A | 36 |
| B | 51 |
| O | 50 |
| AB | 31 |
| Rat | 151 |
| Rabbit | 157 |
| Sheep | 11 |
| Chicken | 7 |
| Pigeon | 2 |
| Toad | 7 |
| Loach | 11 |
| Grass carp | 31 |
| Lip shark | 15 |

Thirteen species of erythrocytes were incubated with amphioxus humoral fluid diluted 1:20 in presence of 10 mM EGTA-Mg-GVB and the ACH50 values were determined as described under "Materials and Methods".

vators for the CH50 titration of grass carp and lip shark sera (Data not shown).

**Target cell phylogeny**

Erythrocytes from species representing mammals, birds, amphibians, and fish were tested to determine if target cells from phylogenetically closer animals would be more easily hemolysed. As shown in Table 1, all RBC tested activated the hemolytic activity of amphioxus humoral fluid to some extent, but the highest activity was obtained when RaRBC was used. No evident relationship was observed between the phylogeny of the target cells and degree of hemolysis. Interestingly, however, rat erythrocytes were excellent target cells.

**Properties of the hemolytic activity**

Chelation with 100 mM EDTA completely depleted the hemolytic activity of amphioxus humoral fluid, while chelation of the humoral fluid in EGTA-Mg did not affect its hemolytic reaction. In addition, the hemolytic activity of the EDTA-treated humoral fluid was partially (32%) restored by adding $Mg^{2+}$ into it, but not by adding $Ca^{2+}$ (Fig. 1). This indicates that $Mg^{2+}$ is required for the hemolytic reaction, while $Ca^{2+}$ is not.

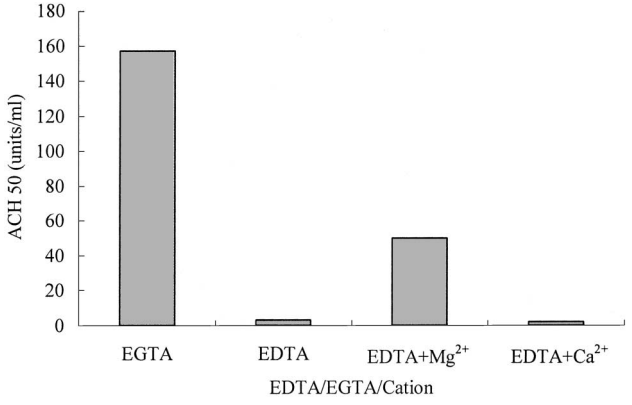


**Fig. 1.** Effects of divalent cations on the hemolytic activity of amphioxus humoral fluid.

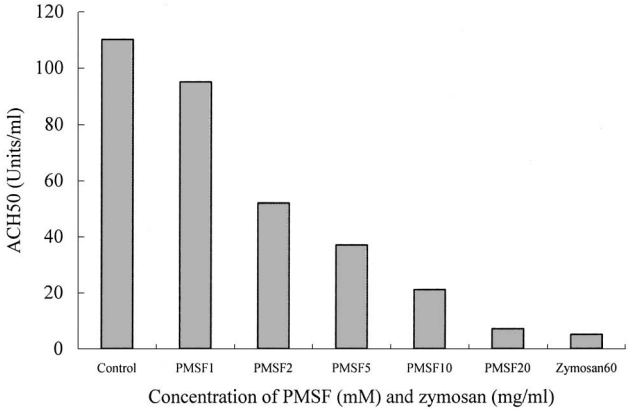


**Fig. 2.** Effects of PMSF and zymosan on the hemolytic activity of amphioxus humoral fluid.

Incubation of amphioxus humoral fluid with zymosan effectively depleted the hemolytic activity. The hemolytic activity of the humoral fluid was also markedly reduced by incubation with PMSF (Fig. 2).

As shown in Fig. 3, small nucleophiles such as hydrazine and methylamine were capable of abrogating the hemolytic activity of amphioxus humoral fluid. The effect of methylamine and hydrazine on the hemolytic activity of amphioxus humoral fluid was concentration-dependent. These indicate the presence of at least one methylamine- and hydrazine-sensitive component in the hemolytic system of the humoral fluid.

Incubation of rabbit anti-human C3 serum with amphioxus humoral fluid abrogated its hemolytic activity, while incubation of normal rabbit serum or PBS with the humoral fluid did not impair the hemolytic activity (Fig. 4). Western blotting analysis demonstrated that like human serum, amphioxus humoral fluid cross-reacted with rabbit anti-human C3 serum, forming a main band equivalent to C3 and two minor bands resembling C3 $\alpha$ and $\beta$ chain, respectively(Fig. 5). These show that C3 component is responsible for the hemolytic activity of amphioxus humoral fluid.

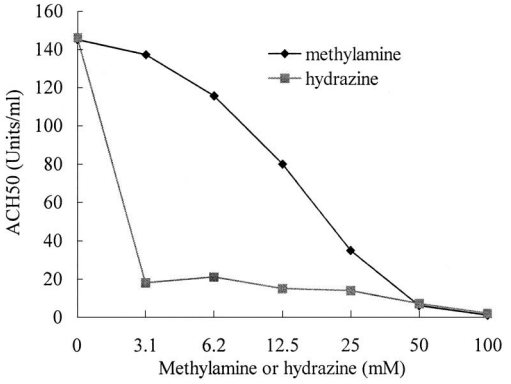


**Fig. 3.** Effects of methylamine and hydrazine on the hemolytic activity of amphioxus humoral fluid.

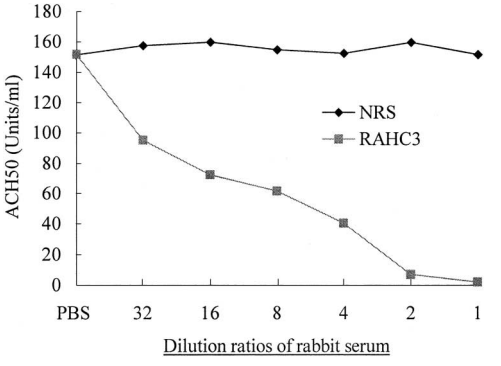


**Fig. 4.** Effects of rabbit anti-human C3 (RAHC3) and non-immune rabbit sera (NRS) on the hemolytic activity of amphioxus humoral fluid. Aliquots of 50 µl of the serially diluted antisera were mixed with an equal volume of amphioxus humoral fluid, and the mixtures were incubated at 25°C for 1 hr. After centrifugation at 3 000 g for 10 min, the supernatants were diluted 10-fold with 10 mM EGTA-Mg-GVB, and their hemolytic activity was determined.

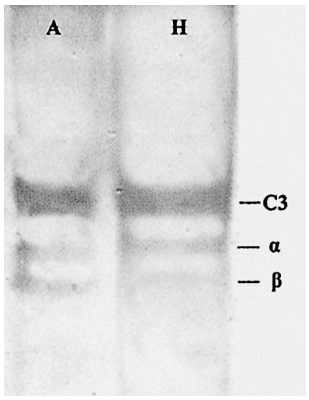


**Fig. 5.** Western blotting of C3 in amphioxus humoral fluid. Amphioxus humoral fluid and human serum were electrophoresed on 7.5% native PAGE. Proteins separated were transferred onto nitrocellulose membrane and immunostained with rabbit anti-human C3 serum. H, human serum; A, amphioxus humoral fluid.

The content of C3 component in amphioxus humoral fluid was then quantified by TIA. It was found that the concentration of C3 component in amphioxus humoral fluid ranged from 0.79 mg/ml to 1.47 mg/ml with the average of 1.17 mg/ml, which was close to C3 concentration in human sera (0.98 mg/ml).

These results exhibit that the complement-like activity is present in the cephalochordate amphioxus.

**Optimal conditions for ACH50 assay**

Amphioxus humoral fluid showed the highest hemolytic activity at 20°C, and even at 4°C a considerable amount of hemolysis was observed (Fig. 6). After incubation at 37°C for 90 min, the ACH50 titer decreased to about 50% of the highest value. Heating at 45, 50 and 56°C completely depleted the hemolytic activity. It is clear that the hemolytic activity is heat-labile.

The optimum pH for the hemolytic reaction of amphioxus humoral fluid was 7.5 and at pH values lower than 6.5 or higher than 9, the hemolytic activity was significantly reduced (Fig. 7).

The optimum concentration of $Mg^{2+}$ for the hemolytic reaction of amphioxus humoral fluid was 10 mM (Fig. 8).

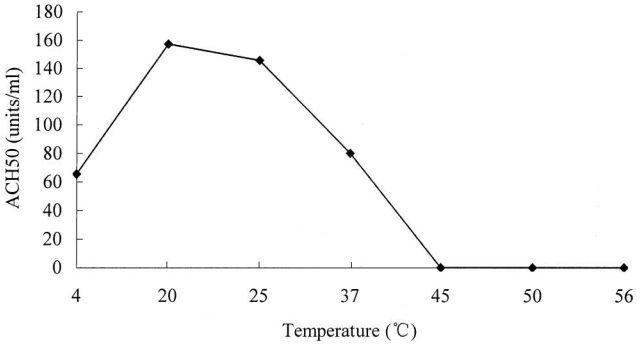


**Fig. 6.** Effect of temperature on the hemolytic activity of amphioxus humoral fluid.

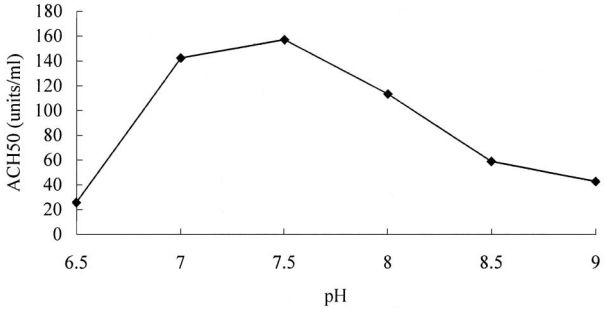


**Fig. 7.** Effect of pH on the hemolytic activity of amphioxus humoral fluid.

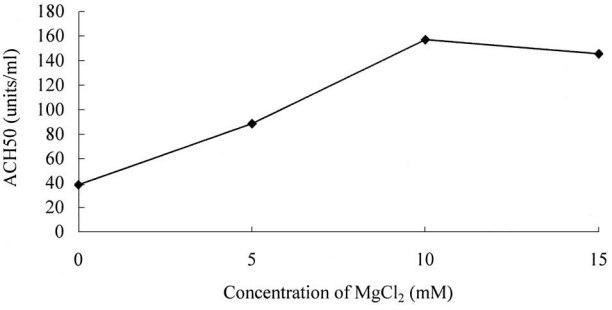


**Fig. 8.** Effect of the concentration of $MgCl_2$ on the hemolytic activity of amphioxus humoral fluid.

Notably, some hemolytic activity was observed at 0 mM $Mg^{2+}$, which might be attributable to $Mg^{2+}$ originally present in the humoral fluid.

## DISCUSSION

The standard hemolytic analysis method of Yano (1992) has been applied to the assay of hemolytic complement activity in amphioxus humoral fluid in the present work. We have demonstrated for the first time the presence of a hemolytically active complement system in amphioxus humoral fluid. Evidences indicating the complement nature of the hemolytic activity of amphioxus humoral fluid include: (1) ability of the humoral fluid to hemolyse not only RaRBC but also heterologous erythrocytes suspended in EGTA-Mg-GVB rather than sensitized SRBC suspended in $GVB^{2+}$; (2) requirement of $Mg^{2+}$ rather than $Ca^{2+}$; (3) abrogation of the hemolytic activity by pre-incubation of amphioxus humoral fluid with known inactivators of the mammalian AP such as zymosan, methylamine and hydrazine; (4) depletion of the hemolytic activity by incubation of amphioxus humoral fluid with rabbit anti-human C3 serum, but not incubation with normal rabbit serum; (5) presence of C3 component in the humoral fluid; and (6) inhibition of the hemolytic activity by heat or EDTA. These properties are apparently similar to those observed in the complement system of the vertebrates including fish and mammals (Vogel and Müller-Eberhard, 1985; Sunyer and Tort, 1995), and indicate that the CP is not present in amphioxus. This is also in line with the

hypothesis that the CP appears with the evolutionary emergence of the jawed vertebrates (Smith *et al*., 1999; Zarkadis *et al*., 2001; Lambris *et al*., 1999).

Recently, the mannose-binding lectin-associated serine protease (MASP) (Ji *et al*., 1997), and C3 (Nonaka *et al*., 1999) have been identified in the invertebrates like urochordate, and the origin of lectin-dependent pathway is traced back to the invertebrates (Sekine *et al*., 2001). Therefore, the possibility cannot be ruled out that the lectin pathway is present in amphioxus. However, the facts that the hemolytic activity displayed by amphioxus humoral fluid does not require $Ca^{2+}$ and is inhibited by pre-incubation with the known specific inactivators of the mammalian AP, zymosan, methylamine and hydrazine, suggest that the hemolytic activity is probably due to the complement system operating via the AP. Detailed molecular study of the hemolytic activities in amphioxus will aid elucidating the evolutionary relationship between AP and lectin pathway.

In general, the levels of complement are expressed in ACH50 and CH50 titers, which are defined as the reciprocal of the serum dilution causing 50% hemolysis of unsensitized RaRBC and sensitized SRBC, respectively. The ACH50 titers of human, cattle, pig, sheep, dog, guinea pig and rat sera are 18.4, 43.0, 13.6, 15.4, 14.4, 11.9 and 4.5 units/ml, respectively (Vogel and Müller-Eberhard, 1976), and those of porgy, yellowtail, eel and sea bream sera 92, 142, 134 and 170 units/ml, respectively (Matsuyama *et al*., 1988; Sunyer and Tort, 1995). The ACH50 observed in amphioxus humoral fluid is much higher than that of mammals, but approximates that of fish. This suggests a greater importance for the AP in fish and amphioxus than in higher vertebrates. It is of interest to note that the CP system is only poorly evolved in fish when compared with that of mammals. As amphioxus lacks the CP, it is conceivable that the AP functions as an important non-specific defense mechanism in this animal.

Suzuki *et al*. (2002) have recently cloned a C3-like gene fragment from amphioxus, which is structurally similar to vertebrate C3. It is revealed here by means of Western blotting and TIA that there exists a C3 component in amphioxus humoral fluid. The presence of the C3 component is further supported by the fact that incubation of rabbit anti-human C3 serum, and C3-depleting and C3-destroying agents with amphioxus humoral fluid abrogates its hemolytic activity. These together show that C3 component is involved in the hemolytic activity of amphioxus humoral fluid, and amphioxus C3 component shares at least partial antigenicity with human C3. The content of C3 component in amphioxus humoral fluid (1.17 mg/ml) is comparable to C3 concentration in human (0.8 to 1.3 mg/ml) and dog (1.24±0.32 mg/ml) sera (Tack and Prahl, 1976; Lucena *et al*., 1999).

Amphioxus humoral fluid displays hemolytic activity against red blood cells (RBC) from different animals including mammals, birds, amphibians and fish, but there is no clear relationship between the phylogeny of the target cells and degree of hemolysis. RaRBC have been shown the best activators for the AP in higher vertebrates, and RaRBC are also found to be most sensitive to hemolysis by amphioxus humoral fluid, suggesting RaRBC are universally excellent target cells for hemolytic activity assay of both vertebrates and invertebrates. Interestingly, rat RBC are found to be highly sensitive to hemolysis by amphioxus humoral fluid. It is known that the terminal sugars on the glycoproteins on the surface of RBC in addition to some associated serum proteins are important factors to activate the AP. RaRBC are missing sialic acid and therefore make them excellent test cells to assay AP complement activity (Fearon, 1978; Dijk *et al*., 1985; Sunyer and Tort, 1995). Sheep RBC, on the other hand, have a lot of sialic acid, and this is one aspect of their inability to activate the AP and be lyzed by the terminal pathway. Possibly, rat erythrocytes also lack sialic acid on their cell surface, while erythrocytes from human, chick, pigeon, toad, grass carp, loach and lip shark are all sialic acid-rich cells.

Serine protease has been proved an integral component of the mammalian complement system (Reid and Porter, 1981), sea star complement-like system (Leonard *et al*., 1990) and arthropod coagulation system (Nakamura and Levin, 1982). The serine protease activity is also an important component of amphioxus hemolytic system since its hemolytic activity is sensitive to treatment of PMSF.

The present study has also established the standard method for ACH50 titration of amphioxus humoral fluid. The activity is optimally assayed at 20°C, at pH 7.5, and in the presence of 10 mM $Mg^{2+}$. This method will be very useful for further identification of amphioxus hemolytic system and for determination of hemolytic activity in the humoral fluid from other invertebrates.

The optimal pH and $Mg^{2+}$ concentration for ACH50 titration of amphioxus humoral fluid both resemble those observed in higher vertebrates. The optimum temperature for ACH50 titration of amphioxus humoral fluid is also similar to that (20 to 25°C) for fish (Yano, 1992), but it is considerably lower than that (37°C) for mammals. It is noteworthy that the hemolytic activity of amphioxus humoral fluid remains at 4°C, but it is readily inactivated at 37°C. This may be due to that the amphioxus live in sea water where the ambient temperature varies widely, yet seldom rises as high as 37°C.

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## REFERENCES

Al-Sharif WZ, Sunyer JQ, Lambris JD, Smith LC (1998) Sea urchin coelomocytes specifically express a homologue of the complement component C3. J Immunol 160: 2983–2997

Benedictis GD, Capalbo P (1981) An Amphioxus lanceolatus agglutinating factor for human red cells. J Immunogenet 8: 225–230

Davis R (1964) Disc electrophoresis. Method and application to human serum proteins. Ann NY Acad Sci 121: 404–427

Day NKBPHD, Gewurz HMD, Johannsen R, Finsted J, Good RAMD (1970) Complement and complement-like activity in lower veterbrates and invertebrates. J Exp Med 132: 941–950

Dijk VH, Rademaker, PM, Klerk JPAM, Williers JMN (1985) Study of the optimal reaction conditions for assay of the mouse alternative complement pathway. J Immunol Methods 85: 233–243

Fearon DT (1978) Regulation by membrane sialic acid of B1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. Proc Natl Acad Sci USA 75: 1971–1975

Gotze O, Müller-Eberhard HJ (1976) The alternative pathway of complement activation. Adv Immunol 24: 1–35

Hanley PJ, Hook JW, Raftos DA, Gooley AA, Trent R, Raison RL (1992) Hagfish humoral defense protein exhibits structural and functional homology with mammalian complement components. Proc Natl Acad Sci USA 89: 7910–7914

Ji X, Azumi K, Sasaki M, Nonaka M (1997) Ancient origin of the complement lectin pathway revealed by molecular cloning of mannan binding protein-associated serine protease from a urochordate, the Japanese ascidian, *Halocynthia roretzi*. Proc Natl Acad Sci USA 94: 6340–6345

Kai C, Yashikawa Y, Yamanouchi K, Okada H (1983) Isolation and identification of the third component of Japanese quails. J Immunol 130: 2814–2820

Koppenheffer TL (1987) Serum complement system of ectothermic vertebrates. Dev Comp Immunol 11: 279–286

Lambris JD, Reid KBM, Volanakis JE (1999) The evolution, structure, biology and pathophysiology of complement. Immunol Today 20: 207–211

Leonard LA, Strandberg JD, Winkelstein JA (1990) Complement-like activity in the sea star, *Asterias forbesi*. Dev Comp Immunol 14: 19–30

Li GR, Zhang SC, Xiang JH (2000) Phenoloxidase, a marker enzyme for differentiation of the neutral ectoderm and the epidermal during embryonic development of amphioxus *Branchiostoma belcheri tsingtauense*. Mech Develop 96: 107–109

Lowry PH, Rosebrough NJ, Farr L, Randall RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193: 256–275

Lucena R, Ginel P. J, Hernandez E, Novales M (1999) Effects of short courses of different doses of prednisone and dexamethasone on serum third component of complement (C3) levels in dogs. Vet Immunol Immunopathol 68: 187–192

Matsuyama H, Tanaka K, Nakao M, Yano T (1988) Characterization of the alternative complement pathway of carp. Dev Comp Immunol 12: 403–408

Mock A, Renwrantz L (1987) Lectin activity and a protease inhibitor in mucus from the skin of *Branchiostoma lanceolatum*. J Invertbr Pathol 49: 221–222

Moller PC, Philpott CW (1973) The circulatory system of amphioxus (*Branchiostoma floridae*) I. Morphology of the major vessels of the pharyngeal area. J Morphol 139: 389–406

Nakamura S, Levin J (1982) Endotoxin-mediated Limulus proclotting enzyme activator and detection of a previously undescribed protease (protease N). Biochem Biophys Res Commun 108: 1619–1623

Nonaka M, Azumi K, Ji X, Namikawa-Yamada C, Sasaki M, Saiga H, Dodds AW, Sekine H, Homma MK, Matsushita M, Endo Y, Fujita T (1999) Opsonic complement component C3 in the solitary ascidian, *Halocynthia roretzi*. J Immunol 162: 387–391

Nonaka M, Fujii T, Kaidoh T, Natsuume-Sakai S, Nonaka M, Yamaguchi N, Takahashi M (1984a) Purification of a lamprey complement protein homologous to the third component of the mammalian complement system. J Immunol 133: 3242–3249

Nonaka M, Iwaki M, Nakai C, Nozaki M, Kaidoh T, Nonaka M, Natsuume-Sakai S, Takahashi M (1984b) Purification of a major serum protein of rainbow trout (*Salmo gairdeneri*) homologous to the third component of mammalian complement. J Biol Chem 259: 6327–6333

Ohno S (1970) Evolution by gene duplication. Springer-Verlag, Heidelberg

Ohta H, Kai C, Yashikawa Y, Yamanouchi K (1983) Activation of chicken alternative complement pathway by fowlpox virus-infected cells. Infec Immun 42: 721–727

Reid KBM, Porter RR (1981) The proteolytic activation systems of complement. Annu Rev Biochem 50: 433–464

Ruddy S (1974) The immunobiology of complement. Chemistry and biologic activity of the complement system. Trans Proc 6: 1–7

Sekine H, Kenjo A, Azumi K, Ohi G, Takahashi M, Kasukawa R, Ichikawa N, Nakata M, Mizuochi T, Matsushita M, Endo Y, Fujita T (2001) An ancient lectin-dependent complement system in an ascidian: novel lectin isolated from the plasma of the solitary ascidian, *Halocynthia roretzi.* J Immunol 167: 4504–4510

Sekizawa A, Fujii T, Katagiri C (1984) Isolation and characterization of the third component of complement in the serum of the clawed frog, *Xenopus laevis*. J Immunol 133: 1436–1443

Silva JR, Mendes EG, Mariano M (1995) Wound repair in the amphioxus (*Branchiostoma platae*), an animal deprived of inflammatory phagocytes. J Invertebr Pathol 65: 147–151

Smith LC, Azumi K, Nonaka M (1999) Complement systems in invertebrates. The ancient alternative and lectin pathways. Immunopharmacology 42: 107–120

Smith LC, Shih CS, Dachenhausen SG (1998) Coelomocytes express SpBf, a homologue of factor B, the second component in the sea urchin complement system. J Immunol 161: 6784–6793

Suner Q, Tort L (1994) The complement system of the teleost fish *Sparus aurata.* Ann New York Acad Sci 712: 371–373

Sunyer JQ, Tort L (1995) Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are affected by the alternative complement pathway. Vet Immunol Immunopathol 45: 333–345

Suzuki MM, Satoh N, Nonaka M (2002) C6-like and C3-like molecules from the cephalochordate, amphioxus, suggest a cytolytic complement system in invertebrates. J Mol Evol 54: 671–679

Tack BF, Prahl JW (1976) Third component of human complement; Purification from plasma and physicochemical characterization. Biochemistry 15: 4513–4521

Takada Y, Arimoto Y, Mineda H, Takada A (1978) Inhibition of the classical and alternative pathway by amino acids and their derivatives. Immunology 34: 509–515

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354

Van Dijk H, Rademaker PM, Willers JMN (1980) Estimation of classical pathway of mouse complement activity by use of sensitized rabbit erythrocytes. J Immunol Methods 39: 257–268

Vogel C-W, Müller-Eberhard HJ (1985) The cobra complement system. I. The alternative pathway of activation. Dev Comp Immunol 9: 311–325

Vogel CW, Smith CA, Müller-Eberhard HJ (1984) Cobra venom factor: structural homology with the third component of human complement. J Immunol 133: 3235–3241

Wang CF, Zhang SC, Lu Y, Xu TT (2002) Presence and induction by bacteria of D-galactoside-specific lectins in the humoral fluids of amphioxus *Branchiostoma belcheri tsingtauense.* Inflammopharmacology 9: 241–248

Yano T (1992) Assays of hemolytic complement activity. In “Tech-

niques in Fish Immunology" Ed by Stolen JS, Fletcher TC, Anderson DP, Kaattari SL, Rowley AF, SOS Publication, Fair Haven, pp 131–142

Zarkadis IK, Mastellos D, Lambris JD (2001) Phylogenetic aspects of the complement system. Dev Comp Immunol 25: 745–762

Zhang SC, Li GR (2000) Presence of phenoloxidase and prophenoloxidase in the epidermal cells and epidermis mucus of the lancelet *Branchiostoma belcheri tsingtauense*. Ophelia 52: 207–212

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