Temporal and Spatial Profiles of Alkaline Phosphatase Activity During Embryogenesis of Amphioxus Branchiostoma belcheri tsingtaunese

Authors: Zhang, Shicui, and Wang, Rui

Source: Zoological Science, 18(1) : 51-55

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.51

ZOOLOGICAL SCIENCE **18**: 51–55 (2001)

Temporal and Spatial Profiles of Alkaline Phosphatase Activity During Embryogenesis of Amphioxus *Branchiostoma belcheri tsingtaunese*

Shicui Zhang* and Rui Wang

Department of Marine Biology, Ocean University of Qingdao Qingdao 266003, P. R. China

ABSTRACT—Temporal and spatial profiles of alkaline phosphatase (ALP) activity during the development of amphioxus have not been fully documented and thus the present study investigated this activity spectrophotometrically, electrophoretically and histochemically. The following results were observed: (1) spectrophotometrically, ALP activity increased markedly at the late gastrula stage and reached a plateau at 15 hr postfertilization; (2) the electrophoretic pattern of ALP isozymes changed dramatically during development; (3) ALP activity was initially localized in the posterior wall of the primitive gut and the anterior 5 to 6 somites at about the 15 hr larva stage, and then in the notochord and all the somites at about the 18 hr larva stage; (4) in 1-day larvae, ALP activity decreased in the posterior wall of the primitive gut and in the anterior 5 to 6 somites which had ALP activity at 15 hr, but it appeared in the newly formed somites, especially in the myosepta, the crevices cut in between adjoining somites; (5) in 2-day larvae, ALP activity was no longer visible in somites but became highly evident in most of the notochord except for its rostral region; and (6) when the lateral plate mesoderm pushed down ventrally on either side of the intestine and conjoined beneath the intestine, ALP activity was also detected in the conjoining lateral plate mesoderm. Apparently, two types of ALP exist in amphioxus larvae, the transient endodermal ALP and the constant mesodermal ALP, and the spatial and temporal correlation of ALP activity with the developing mesoderm, including the notochord, suggests that it plays a role in the differentiation of mesodermal structures during the development of amphioxus.

INTRODUCTION

Alkaline phosphotase (ALP: orthophospheric-monoester phosphohydrolase, EC3.1.3.1) exists in both animals and plants as well as microorganisms. It is a highly glycosylated membrane-bound enzyme and catalyzes hydrolysis of inorganic and organic phosphate esters with an alkaline pH *in vitro*. The development of ALP has thus far been studied in a number of animal embryos including those of ascidians (Whittaker, 1977; Whittaker,1990), amphibians (Maekawa and Yamana,1975), chicks (Moog, 1944) and mammals (Kwong and Tam, 1984; Rossi and Reale, 1984; Tam and Snow, 1981). It has been shown that the first sharp increase in ALP activity occurs at about the gastrula stage during the development of ascidian and amphibian embryos. The onset of ALP activity and its localization suggest that it plays a role in the course of cell differentiation and morphogenesis. In mice, strong ALP activity has been found in the somitic mesoderm, intestinal epithelium and migrating primordial germ cells at the early organogenetic stage (Rossi and Reale, 1957; Tam and Snow, 1981). In ascidians, ALP has been found in the endoderm at the gastrula stage and has thus been used as a histochemical marker for endodermal or gut-cell differentiation (Whittaker, 1977; Whittaker,1990).

The amphioxus or lancelet, a cephalochordate, has long been regarded as the living invertebrate most closely related to vertebrates. ALP has been purified from amphioxus, *Branchiostoma belcheri* and studied enzymologically by Yan *et al.* (Yan *et al.*, 1980; Yan *et al.*, 1981; Yan and Chen, 1985) and Chen and Chen (Chen and Chen, 1989). However, little is known about the temporal and spatial profiles of ALP activity in amphioxus embryos and larvae. This study addresses the temporal and spatial profiles of ALP activity during the embryogenesis of amphioxus.

MATERIALS AND METHODS

Amphioxus

Amphioxus *Branchiostoma belcheri tsingtaunese* were collected during the breeding season from the sandy bottom of the sea near Shazikou, Qingdao, and cultured in containers with continuous aeration. Fresh seawater and unicellular algae were supplied daily. Spawning of the animals was checked every evening until the spawning

* Corresponding author: E-mail: zscshab@public.qd.sd.cn

season was over. Fertilized eggs were collected from the animals and cultured in filtered seawater (Wu *et al.*, 1994). Embryos and larvae were collected at desired stages and rinsed three times with 50 mM Tris-HCl containing 100 mM NaCl, pH7.5. Rinsed embryos and larvae were either stored at –20°C for enzyme assay and electrophoresis or fixed with 4% paraformaldehyde in 100 mM MOPS (pH7.5) for histochemical staining.

Enzyme preparation

Rinsed embryos and larvae were homogenized in about one volume of 50 mM Tris-HCl with 100 mM NaCl (pH7.5). The homogenate was mixed with 0.3 volume of n-butanol and maintained at 37 °C for 30 min (Morton, 1954). After centrifugation at 2, 000 g for 15 min at room temperature, the supernatant was pooled. Protein concentrations were determined by the method of Lowry *et al.* (Lowry *et al.*, 1951).

Enzyme activity assay

The enzyme activity was measured by the method of Lowry (Lowry, 1957). Briefly, 50 μl of the supernatant was mixed with 300 μl of 8 mM p-nitrophenyl phosphate (Sangon, Ameresco) in 500 mM 2-amino-2-methyl-1-propanol (Sangon, Ameresco) buffer (pH10.2) containing 2 mM $MgCl_2$. The mixture was incubated in darkness for 30 min at 37°C. After addition of 900 μl of 250 mM NaOH, absorption at 410 nm was measured. Standards and blanks consisting of 10 μl of 1 mM p-nitrophenol or double distilled water were carried through the entire procedure with the supernatant. The enzyme activity was expressed as units, where one unit was defined as μM of p-nitrophenyl phosphate hydrolyzed per min per milligram of protein.

Polyacrylamide gel electrophoresis

Electrophoresis was performed according to the method of Davis (Davis, 1964). Twenty μl of the supernatant mixed with 20 μl of sample buffer (20% glycerol-0.05% bromphenol blue-125 mM Tris-HCl, pH6.8) was loaded on the discontinuous gel, which consisted of a 3% spacer gel and a 7.5% separation gel, and run at room temperature until the bromphenol blue marker had migrated to the end of the gel. The enzyme activity was detected by a slightly modified version of Burstone's (Burstone, 1962) method. The gel was incubated in a reaction medium consisting of 2 mM α-naphthyl phosphate (Sigma, St. Louis), 1.8 mM fast red TR (Sigma, St. Louis), 2 mM $MgCl_2$ and 10 mM Tris-HCl (pH10) for 30 min at room temperature and then washed three times in double distilled water.

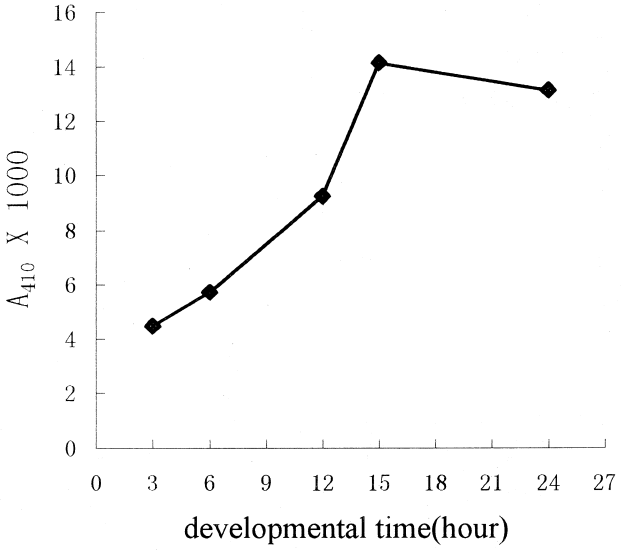

Fig. 1. Change in ALP activity during embryogenesis of amphioxus.

Histochemistry

The fixed embryos and larvae were rinsed with 100 mM Tris-HCl (pH9.6), 500 mM $MgCl_2$ and 100 mM NaCl and incubated in the reaction medium of 2 mM sodium α-naphthyl phosphate, 1.8 mM fast red TR, 2 mM $MgCl_2$ and 10 mM Tris-HCl (pH10) for 10 min at room temperature. Control for the histochemical staining was performed either by adding 1 mM tetramisole hydrochloride (Sigma, St. Louis) or 10 mM L-phenylalanine to the reaction medium to inhibit the enzyme, or by removing the substrate from the medium.

RESULTS

Changes in ALP activity

As shown in Fig.1, ALP activity was spectrophotometrically constant and comparatively weak during the first 3.5 or 6 hr, i.e., at the blastula and mid-gastrula stages. The enzyme activity increased markedly at the late gastrula stage and reached a plateau at 15 hr postfertilization when 5 to 6 somites were formed.

Changes in patterns of ALP isozymes

ALP isozymes migrated anodally. As shown in Fig. 2, 5 ALP isozyme bands were seen on the gel, i.e., ALP1, ALP2, ALP3, ALP4 and ALP5, with ALP1 being the fast moving form and ALP5 the slowest moving one. ALP isozymes were not detected at the blastula and gastrula stages but first became electrophoretically detectable at the neurula stage at about 10 hr postfertilization. Initially, 4 bands, ALP1, ALP2, ALP3

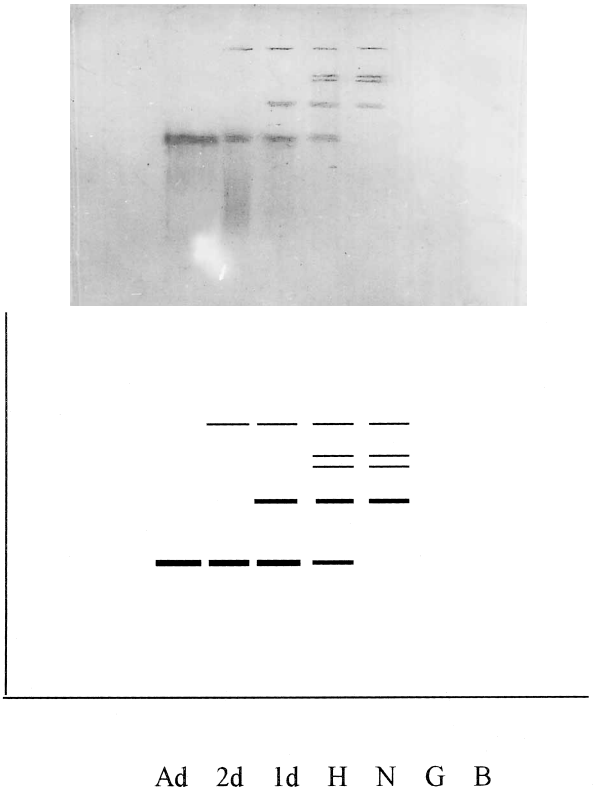

Fig. 2. Electrophoresis diagram of ALP isozymes during embryogenesis of amphioxus. Ad: adult; 2d: 2-day larva; 1d: 1-day larva; H: hatched larva; N: neurulae; G: gastrulae; B: blastulae.

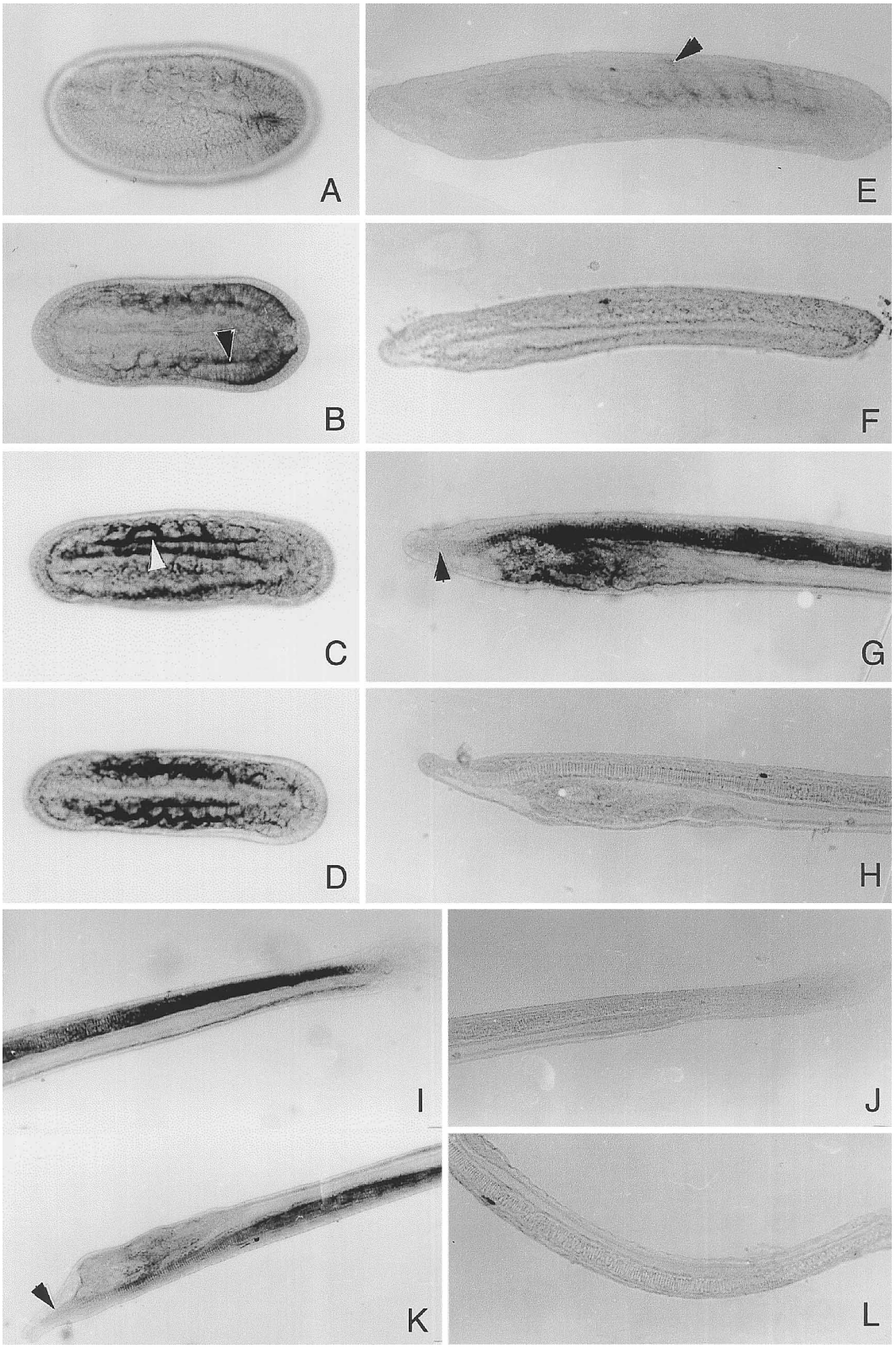

Fig. 3. Localization of ALP activity in the amphioxus embryos and larvae. Anterior is to the left in all the panels. A (side view) and B (dorsal view): a 15 hr embryo. Note the ALP activity in the somites and the posterior wall of primitive gut (arrowhead). C (side view) and D (dorsal view): an 18 hr embryo. Note the ALP activity in the notochord (arrowhead). E (side view) and F (side view, control): 1-day larvae. Note the presence of ALP activity in the myosepta in E but its absence in F. G and I (left-side view) and K (right-side view): 2-day larvae. Note the absence of ALP activity in the rostral region of the notochord (arrowhead). H and J (left-side view) and L (right-side view) are controls incubated in the absence of the substrate, sodium α-naphthy1 phosphate (H and J) or in the presence of tetramisole hydrochloride (L). A, B, C, D and E: × 318; F: × 140 G, H: × 159; I, J, K and L: × 120.

and ALP4, were observed in the developing neurulae, and another new band, ALP5, was seen in the hatched larvae, when the first 2 to 3 somites were formed at about 12 hr postfertilization. However, ALP2–3 and ALP4 soon disappeared in 1-day and 2-day larvae, respectively. In contrast, only ALP5 remained evident in adult amphioxus. Clearly, marked changes took place in the pattern of ALP isozymes during the embryogenesis of amphioxus.

Localization of ALP

ALP activity was completely inhibited by the addition of tetramisole hydrochloride to the incubation medium or by removal of the substrate from the medium (Fig. 3 F, H, J, L). When L-phenylalanine was added to the incubation medium, the enzymatic reaction was also markedly reduced (data not shown). This indicated that the activity of ALP was responsible for the staining reaction.

Histochemically, no ALP activity was detected in embryos earlier than 15 hr after fertilization. ALP activity was initially observed in the first 5 to 6 somites and the posterior wall of the primitive gut in 15 hr embryos and then in the posterior wall of the primitive gut, somites and notochord in about 18 hr embryos (Fig. 3A, B, C, D). As embryos were elongated and somites formed in succession from the anterior part backwards, ALP activity decreased in the posterior wall of the primitive gut and the initial 5 to 6 somites which exhibited ALP at 15 hr, but it appeared in the newly formed somites, especially in the myosepta, the crevices cut in between adjoining somites in 1-day larvae (Fig. 3E). In 2-day larvae, ALP activity was no longer visible in somites; instead, it became highly evident in most of the notochord except for its rostral region (Fig. 3G, K). On the other hand, when the lateral plate mesoderm pushed down ventrally on either side of the intestine and conjoined beneath the intestine, ALP activity was also detected in the conjoining lateral plate mesoderm (Fig. 3G, I, K).

DISCUSSION

The temporal and spatial profiles of ALP activity in the embryos and larvae of amphioxus were studied for the first time in the present study. Spectrophotometrically, ALP activity was found to first increase markedly at the late gastrula stage, which is generally in line with observations of this activity in ascidians and amphibians. However, ALP activity became electrophoretically detectable at the neurula stage at about 12 hr postfertilization when the first 2 to 3 somites were formed and histochemically detectable at 15 hr postfertilization. Thus it was found that for measurement of ALP activity, spectrophotometric assay is more sensitive than electrophoretic analysis, which in turn is more sensitive than histochemical staining.

Histochemical staining showed the presence of two types of ALP, the endodermal ALP and mesodermal ALP, in amphioxus larvae. The mesodermal ALP activity remained located in the mesodermal tissues in 1- and 2-day larvae. However, the endodermal ALP activity was transient in the neurulae and started disappearing before the endoderm underwent differentiation. The significance of the transient presence of ALP activity in the posterior wall of primitive gut is unknown. Interestingly, the constant presence of ALP activity in the mesodermal tissues of amphioxus larvae is in sharp contrast with the fact that ALP is only localized in the endoderm and serves as a histochemical marker of endodermal differentiation during the development of ascidians (Whittaker, 1977; Whittaker,1990). Whether this difference is due to the different species remains to be determined.

The electrophoretic patterns of ALP isozymes changed markedly during the development of amphioxus. Basically, the bands with smaller molecular weights gradually disappeared during development, and only ALP5 remained evident in adults. The relationships between changes in patterns of ALP isozymes, morphogenesis and cell differentiation must be further explored.

The localization of ALP activity in the mesoderm also changed during the embryogenesis of amphioxus. ALP activity was localized in 5 to 6 somites in 15 hr embryos and then in the notochord and newly formed somites in 1-day larvae, and finally in the notochord and conjoining lateral mesoderm beneath the intestine in 2-day larvae. The spatial and temporal correlation of ALP activity with the developing mesoderm, including the notochord, suggests that it might be involved in the differentiation of the mesoderm during the embryogenesis of amphioxus.

It is of interest to note that ALP activity in 2-day larvae was localized in most of the notochord except for its rostral region. One unique feature of amphioxus is that its notochord extends to the extreme anterior tip of the body, beyond the rostral limit of the neural tube. Willey (Willey, 1894) claimed that this rostral region of the amphioxus notochord is slightly retarded in its development, reflecting its later evolution. Shimeld (Shimeld, 1997; Shimeld, 1999) has recently demonstrated a clear difference between the rostral and the other parts of the notochord in *HNF3* and *sonic hedgehog* expression. However, an expression analysis of the amphioxus *Brachyury* gene carried out by Holland *et al.* (Holland *et al.*, 1995) provided no evidence for either a qualitative difference or timing of expression in the rostral and other parts of the notochord. Our observations indicate that in amphioxus, development of the rostral notochord is histochemically distinguishable from that of the remaining part of the notochord, and our observations are in line with Willey's suggestion and Shimeld's results (Shimeld, 1997; Shimeld, 1999). This appears to be the first histochemical evidence demonstrating that the rostral notochord tissue in amphioxus larvae is different from the remaining part of the notochord.

ACKNOWLEDGEMENTS

We would like to thank Dr. Xuecheng Zhang, Ocean University of Qingdao, for his critical reading of the manuscript and Miss Tao Nairong for her technical assistance. We are also very grateful to the anonymous reviewers for their valuable comments and suggestion. This study was supported by a grant from the Natural Science Foun-

dation (No 39770091) of China awarded to S-C.Z.

REFERENCES

Burstone MS (1962) Enzyme histochemistry. New York: Academic Press

Chen QX, SX Chen (1989) Substrate specificity of amphioxus ALPase and the influence of some effectors on the enzyme. Acta Scientiarum Naturalium Universitatis Amoiensis 28: 92–96 (in Chinese)

Davis BJ (1964) Disc electrophoresis. II. Method and application to human serum proteins. Annals N. Y. Acad Sci 121: 404–427

Holland PWH,Koschorz B, Holland LZ, Herrmann BG (1995) Conservation of *Brachyury* (T) genes in amphioxus and vertebrates: developmental and evolutionary implications. Development 121: 4283–4291

Kwong WH, Tam PPL (1984) The pattern of alkaline phosphatase activity in the developing mouse spinal cord. J Embryol Exp Morp 82: 241–251

Lowry OH,Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

Lowry OH (1957) Micromethods for the assay of enzymes. In: Methods in Enzymology. Vol. IV. Academic Press. New York. pp 371–372

Maekawa H, Yamana K (1975) Alkaline phosphatase isozymes of *Xenopus laevis* embryos and tissues. J Exp Zool 192: 155–164

Moog F (1944) Localization of alkaline and acid phosphatases in the early embryogenesis of the chick. Biol Bull 86: 51–80

Morton RK (1954) The purification of alkaline phosphatases of animal tissues. Biochem J 57: 595–603

Rossi F, Reale E (1957) The somite stage of human development studied with the histochemical reaction for the demonstration of alkaline phosphatase. Acta Anat 30: 656–681

Shimeld SM (1997) Characterization of amphioxus *HNF-3* genes: conserved expression in the notochord and floor plate. Dev Biol 183: 74–85

Shimeld SM (1999) The evolution of the *hedgehog* gene family in chordates: insights from amphioxus hedgehog. Dev Genes Evol 209: 40–47

Tam PPL, Snow MHL (1981) Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. J Embryol Exp Morph 64: 133–147

Whittaker JR (1977) Segregation during cleavage of a factor determining endodermal alkaline -phosphatase development in ascidian embryos. J Exp Zool 202: 139–154

Whittaker JR (1990) Determination of alkaline phosphatase expression in endodermal cell lineages of an ascidian embryo. Biol Bull 178: 222–230

Willey A (1894) Amphioxus and the ancestry of the vertebrates. New York: Macmillan

Wu SH, Zhang SC, Wang YY, Bao BL, Qu YM, Jiang XJ (1994) Laboratory observation on spawning, fecundity and larval development of amphioxus (*Branchiostoma belcheri tsingtauense*). Chin J Oceanol Limnol 12: 289–294

Yan SX, Chen SL, Cai HY, Chen XX, Zhang LH (1980) Enzymological studies on *amphioxus Branchiostoma belcheri* (Gray) II: Kinetics of purified alkaline phosphatase. Acta Scientiarum Naturalium Universitatis Amoiensis. 19: 44–71 (in Chinese)

Yan SX, Chen SL, Chen XX, Cai HY, Lin YF (1981) Relationship between functional groups and activity of alkaline phosphatase from amphioxus *Branchiostoma belcheri* (Gray). Acta Scientiarum Naturalium Universitatis Amoiensis. 19: 86–91 (in Chinese)

Yan SX, Chen QX (1985) Preliminary studies on molecular weight and amino acid composition of alkaline phosphatase from amphioxus (*Branchiostoma belcheri)* (Gray). Acta Scientiarum Naturalium Universitatis Amoiensis. 24: 367–372 (in Chinese)

(Received January 5, 2000 / Accepted June 7, 2000)