Purification, Kinetics and Immunochemistry of Two Homotetrameric Lactate Dehydrogenase Isozymes in *Pseudogobio esocinus* (Cypriniformes)

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Three tissues of heart, kidney and liver of a primitive cyprinid *Pseudogobio esocinus* were found to have lactate dehydrogenase isozyme(s) containing subunit C. Tissue expressions of genes for subunits A and B were similar to those of mammalian species. Molecular weight of the isozymes were estimated to be 140,000 approximately.

Affinity chromatography of the isozymes on the immobilized oxamate gel revealed that A_4 isozyme was not eluted in NAD^+ but in column buffer. B_4 isozyme was isozymatically purified by subjecting kidney extract to a CM-Sepharose column. A_4 isozyme as well as B_4 isozyme was inhibited by high concentrations of pyruvate. The affinity chromatographic behavior and susceptibility to pyruvate inhibition of the A_4 isozyme suggest that A_4 isozyme is similar to B_4 isozyme kinetically.

Antibodies against *P. esocinus* A_4 isozyme reacted with mouse A_4 isozyme but not with *P. esocinus* B_4 isozyme, reflecting that subunit B is less conservative in its evolution.

KEY WORDS: Cypriniformes, Lactate dehydrogenase, Subunit C, Oxamate gel, Pyruvate inhibition, Evolution rate

The spatial expression of three distinct structural genes for lactate dehydrogenase (EC. 1.1.1.27; LDH) in teleosts has been a key to understanding the physiological function of isozymes in their cytoplasmic environments (Everse and Kaplan, 1975; Whitt, 1984). It is generally accepted that the ancestral form of LDH closely resembled the present LDH A_4 isozyme and that the gene for the subunit A duplicated to give rise to subunits A and B. The subunit C was then produced by a succession of independent duplication of the gene which coded for the subunit B (Holmes and Scopes, 1974, Markert *et al.*, 1975).

However, by making use of the amino acid sequences of the three homotetrameric LDH isozymes from several species and determining the best possible phylogenetic trees to describe the evolutionary relationship among the LDH isozymes, Rehse and Davidson (1986a) presented an evidence that subunit C rather than subunit A was the ancestral form of vertebrate LDH isozymes and that a duplication of the gene for this subunit C gave rise to gene, which, by a further gene duplication, yielded the subunit A and B genes.

Those two radically different views of LDH isozyme evolution seem to require biochemical reevaluations of LDH A_4 and B_4 isozymes from teleost species in which tissue expressions of all three genes could be detected. In the present work with LDH isozymes in a cyprinid species, we have observed that A_4 isozyme is similar to B_4 isozyme kinetically but not immunochemically.

Materials and Methods

Materials

Pseudogobio esocinus was collected at the upper part of the Keum River (Shintanjin, Korea) and carried to the laboratory alive. Albino male mice (ICR) were used in eliciting antibodies. Bovine heart tissue was used in determining molecular weight of LDH isozymes.

NAD^+, NADH, nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), CM-Sepharose, w-aminohexyl-sepharose 4B, 1-ethyl-3-(3-di methylaminopropyl) carbodiimide were purchased from Sigma. Acrylamide and N, N′-methyl-bis-acrylamide were from Eastman. Sephadex G-200 and Freund's complete adjuvant were from Pharmacia and Difco, respectively.

Preparation of tissue extracts

From freshly killed *P. esocinus* were dissected seven tissues of skeletal muscle, kidney, heart, liver, eye, brain and gill. Each tissue was ground in three volumes (w:v) 0.1 M potassium phosphate buffer (pH 6.85) using an ice-bathed glass homogenizer followed by centrifugation at 25,000 g for 1 hr in a refrigerated centrifuge (Hitachi 20PR-52D). The resulting supernatants were used as LDH extracts.

Determinations of LDH enzyme activity and protein concentration

LDH activity assays were carried out in 0.1 M potassium phosphate buffer (pH 6.85) containing 1.50 mM pyruvate and 0.13 mM NADH. The change in absorbancy at 340 nm was measured in a spectrophotometer (Hitachi 200-20). Protein content of the extracts was determined according to the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

Electrophoresis was performed by employing an acrylamide vertical slab system (LKB 2001), maintained at 4°C by a thermostatic circulator (LKB 2219). Gel plate consisted of 2.5 % stacking gel and 7.5 % separation gel. Samples with sucrose and bromophenol blue were run on the gel plate at 200 V for 1 hr and then at 300 V for 2½ hrs in 0.1 M Tris-glycine buffer (pH 8.3). To locate the enzymatic activity on the gel, the gel was incubated at 37°C with a mixture of NAD^+, DL-lactate, PMS and NBT and then fixed in 15 % acetic acid. To locate the protein zones, the gel was stained with silver nitrate by the method of Wray et al. (1981). Zymograms after the electrophoresis were subjected to densitometry with a laser densitometer (LKB 2202).

Sephadex G-200 gel filtration

To determine the molecular weight of *P. esocinus* LDH, the mixture of 0.5 ml tissue extract of *P. esocinus* skeletal muscle and 0.5 ml bovine heart extract were applied to the Sephadex G-200 column (15 × 270 mm), and the column were eluted with 0.1 M potassium phosphate buffer (pH 6.85). Fractions (2 ml) were assayed for LDH activity and analyzed by polyacrylamide gel electrophoresis.

Affinity chromatography on the oxamate gel

Column preparation for affinity chromatography of LDH isozymes was conducted as described by O'Carra and Barry (1972) and O'Carra et al. (1974) with slight modifications. In brief, aminohexyl-sepharose 4B was swelled in 0.5 M NaCl and activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and potassium oxamate. The column (25 × 92 mm) was equilibrated in the 0.05 M potassium phospate buffer (pH 6.85) containing 0.5 M KCl (the column buffer).

Just before a chromatography, the column was reequilibrated in the column buffer with 0.2 mM NADH. Ten ml of *P. esocinus* skeletal muscle extract containing 0.5 M KCl and 0.2 mM NADH was layered on the column bed, and the column buffer with NADH was applied until no protein was met in effluent. Fractions (2 ml) were collected from the column by applying the column buffer with 1.60 mM NAD and the plain column buffer, successively, and subjected to determinations of enzyme activity and protein concentration. LDH A_4 isozyme was purified to homogeneity by this procedure.

CM-Sepharose chromatography

P. esocinus kidney extract was layered on the CM-Sepharose column (15 × 270 mm) equili-

brated in 0.01 M sodium phosphate buffer (pH 6.08) and a stepwise salt gradient was established. Fractions (1 ml) with LDH activity were electrophoresed to find out the purification of LDH B_4 isozyme.

Preparation of antiserum against the purified LDH A_4 isozyme

The purified LDH A_4 isozyme (0.02 mg) was thoroughly mixed with 0.35 ml Freund's complete adjuvant and injected into male mice intraperitoneally every week for 3 weeks. Blood was drawn from tail tip 5 days after the last antigen injection without anticoagulant. Preparation and use of antiserum in Ag-Ab reactivity have been described elsewhere (Park et al., 1979).

Results and Discussion

LDH isozyme containing subunit C in three tissues

It has been accepted that increase in the number of LDH loci has taken place during the evolution of the fishes. While the agnathans and the chondrichthyes are highly suggested to have LDH isozymes consisted of subunits A and B, most of the bony fishes have known to possess a third LDH subunit, subunit C, to assemble tetrameric LDH isozymes (Markert et al., 1975; Fisher et al., 1980).

Fig. 1 demonstrates the electrophoretic patterns of LDH isozymes in P. esocinus tissues. Tissues of skeletal muscle, eye, brain and gill have an electrophoretic pattern similar to that of mammalian species, A_4 isozyme being predominant in skeletal muscle and B_4 isozyme being predominant in brain (Dawson et al., 1964). In addition to those five isozymes, tissues of kidney, heart and liver have isozyme(s) with at least one subunit C.

Tissue expression of the gene for subunit C in teleost could be classified into two modes. In primitive bony fishes, the gene for subunit C is expressed in many tissues with species difference in the predominance of expression in different tissues (Markert et al., 1975). In contrast, the more advanced fishes have a highly restricted tissue expression of the gene; i.e., either in retina (Whitt, 1970; Shaklee et al., 1973) or in liver of Gadiformes and Cypriniformes (Sensabaugh and Ka-
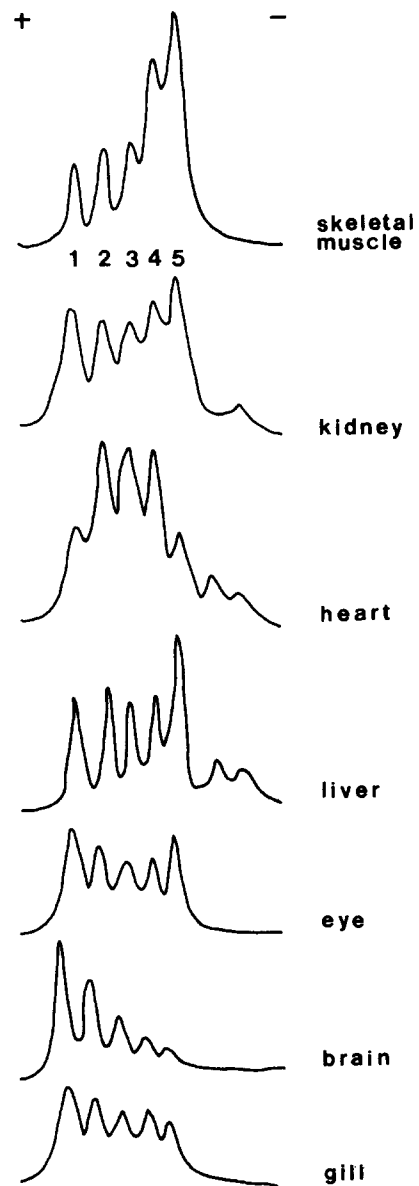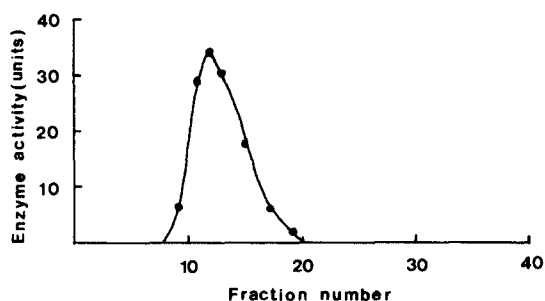

Fig. 1. Densitograms of LDH isozymes in P. esocinus seven tissues. 1, B_4 isozyme; 2, AB_3 isozyme; 3, A_2B_2 isozyme; 4, A_3B isozyme; 5, A_4 isozyme. Kidney, heart and liver tissues appeared to have isozyme(s) containing subunit C at the cathodal side of A_4 isozyme.

plan, 1972; Shaklee et al., 1973; Frankel, 1987).

Kettler and Whitt (1986) proposed a hypothesis of repeated evolutionary canalizations of the gene for subunit C from the generalized tissue expression in more primitive species to a predictable tis-

Table 1. LDH enzyme activity of tissue extracts of *P. esocinus.*

Tissue	Activity (units)	Relative
	Wet tissue (g)	activity (%)
Skeletal muscle	224.1	100.0
Heart	94.5	42.2
Liver	91.6	40.8
Kidney	48.4	21.6
Eye	42.6	19.0
Brain	40.5	18.1
Gill	31.5	14.1

Fig. 3. Polyacrylamide gel electrophoresis of LDH isozymes in (a) fraction 12 after the gel filtration (Fig. 2), (b) bovine heart extract and (c) *P. esocinus* skeletal muscle extract.
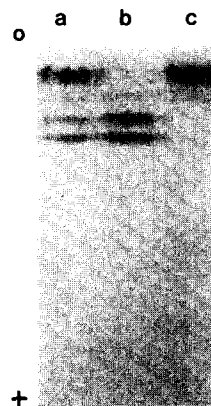

Fig. 2. Gel filtration of LDH isozymes in the mixture of *P. esocinus* skeletal muscle extract and bovine heart extract on a column (15 × 270 mm) of Sephadex G-200.

sue-restricted expression in advanced species. Thus, the occurrence of subunit C in at least three tissues of *P. esocinus* might provide an evidence for the proposed canalization of the gene.

LDH enzyme activities of *P. esocinus* seven tissues shows a relatively wide range, that of the skeletal muscle being the highest (Table 1). It should be noted, however, that the highest LDH enzyme activity could be encountered in liver tissue of salmon (Gesser and Sundell, 1971).

Possible molecular weight of the isozymes

To determine the molecular weight of *P. esocinus* LDH isozyme, the mixture of *P. esocinus* skeletal muscle extract and the bovine heart extract was filtrated through a Sephadex G-200 column (Fig. 2). Aliquot of the fraction of highest enzyme activity was electrophoresed together with the separate extracts. Fig. 3 indicates that *P. esocinus* LDH isozymes are similar in their molecular weights to the bovine LDH isozymes which have

been known to be 140,000 approximately (Pesce et al., 1964) and to the teleost LDH isozymes (Bailey and Wilson, 1968; Rehse and Davidson, 1986b).

Behavior of A_4 isozyme on the immobilized oxamate gel

Oxamate is a structural analog of pyruvate which is not catalytically susceptible, but retains the binding characteristics of pyruvate for LDH isozymes (Schwert, 1970). During the chromatography of tissue extract on the immobilized oxamate gel in the presence of NADH, only the LDH isozymes are bound to the gel. When the NADH is subsequently substituted with NAD^+, LDH A_4 isozyme is eluted spontaneously (O'Carra and Barry, 1972) because of the formation of abortive ternery complex, i.e., $LDH-NAD^+$-pyruvate (Wuntch et al., 1969).

In our affinity chromatography of *P. esocinus* LDH isozymes from skeletal muscle on the immobilized oxamate gel, the LDH isozymes are found to be in the fractions of the plain column buffer with almost no LDH activity in the fractions of NAD^+ (Fig. 4). The subsequent electrophoresis of the fractions of the column buffer revealed that only LDH A_4 isozyme is found in fractions 102 to 107. Fig. 5 represents the electrophoretic results
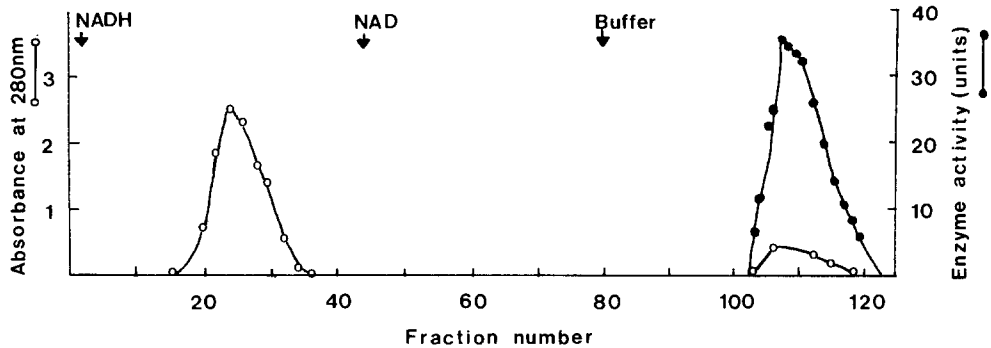
Fig. 4. Elution profile after an affinity chromatography of LDH isozymes in *P. esocinus* skeletal muscle tissue on a column (25 × 92 mm) of immobilized oxamate gel. The LDH A_4 isozyme was found to be in the fractions of the plain column buffer.

Fig. 5. Polyacrylamide gel electrophoresis of LDH isozymes in (a) *P. esocinus* skeletal muscle extract, (b) the purified A_4 isozyme after the affinity chromatography (fraction 106) and (c) the same purified isozyme stained with silver nitrate.

of fraction 106.

This chromatographic results have high reproducibility and provide a basis of postulation that the kinetic behavior of *P. esocinus* A_4 isozyme is similar to that of B_4 isozyme, which is not in agree with the formerly established results from mammalian tissue (O'Carra and Barry, 1972; O'Carra *et al.*, 1974). An evidence for this postulation is discussed in later section.

Purification of B_4 isozyme

CM-Sepharose chromatography was performed to isozymatically purify LDH B_4 isozyme in *P. esocinus* kidney tissue (Fig. 6). Electrophoresis of fractions showing LDH enzyme activity demonstrates that fractions 19 to 22 contain only B_4 isozyme
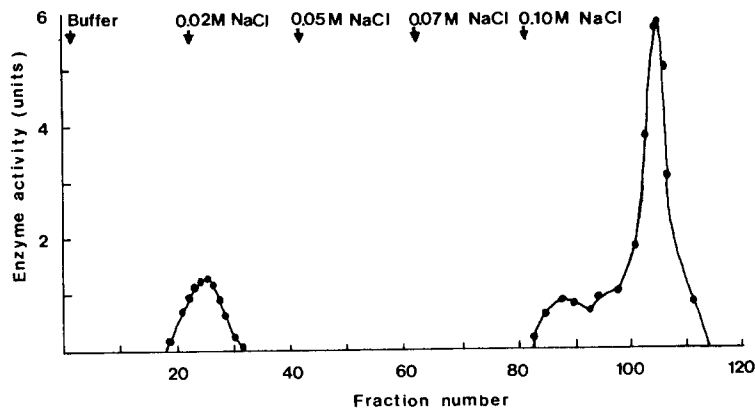

Fig. 6. Elution profile after a CM-Sepharose column (15 × 270 mm) chromatography of LDH isozymes in *P. esocinus* kidney extract. Only B_4 and AB_3 isozymes were found in the first peak.
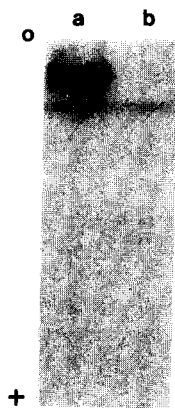
Fig. 7. Polyacrylamide gel electrophoresis of LDH isozymes in (a) *P. esocinus* kidney extract and (b) the purified B_4 isozyme after the CM-Sepharose column chromatography (fraction 22).

Fig. 8. Effect of pyruvate concentrations on the activity of the A_4 and B_4 isozymes of *P. esocinus*. Enzyme activity was measured by following the oxidation of NADH spectrophotometrically at $25°C$ in 0.1 M potassium phosphate buffer, pH 6.85. A_4 isozyme ●-●; B_4 isozyme ○-○. A_4 isozyme appeared to be far more susceptible to the pyruvate inhibition than was the A_4 isozyme of other vertebrate species.

(Fig. 7) and fractions 23 to 31 B_4 and AB_3 isozymes. The other three isozymes were found to be in the fractions of second peak of the elution profile, A_4 isozyme being the last one through the column.

Susceptibility of two homotetramers to pyruvate inhibition

It was noted previously that B_4 isozyme is strongly inhibited by fairly low concentrations of pyruvate, whereas A_4 isozyme functions at the same pyruvate concentrations and is inhibited only by very high pyruvate concentrations (Wilson et al., 1963; Whitt, 1970; Baldwin and Gyuris, 1983). Fig. 8 shows curves obtained by plotting percentage of initial activity against pyruvate concentration. Both A_4 and B_4 isozymes gain the maximal activity at about 1.0 mM pyruvate. B_4 isozyme is inhibited intensively by substrate to such an extent that activity at 10 mM pyruvate is about 28 % of the maximum. At the same substrate concentration, on the other hand, retained activity of A_4 is 50 % of the maximum. This significant decrease in enzyme activity of B_4 isozyme is essentially identical to those reported previously (Whitt, 1970; Baldwin and Gyuris, 1983).

It should be noted, however, that *P. esocinus* A_4 isozyme is far more susceptible to pyruvate inhibition than are A_4 isozymes of other species. Maximal activity of Atlantic hagfish A_4 isozyme at
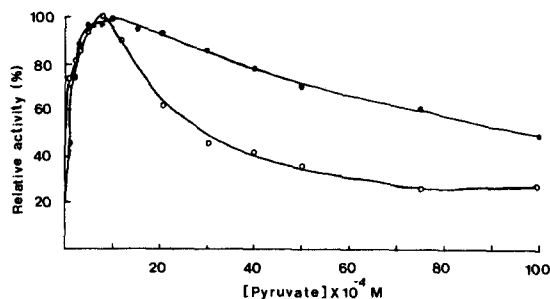
5 mM pyruvate is 92 % (Sidell and Beland, 1980). Furthermore, maximal activity of *P. esocinus* A_4 isozyme at 10 mM pyruvate (50 %) is lower than that of pond turtle A_4 isozyme (80 %) (Altman and Robin, 1969). This unusual high susceptibility of A_4 isozyme to pyruvate inhibition might substantiates the result that the *P. esocinus* A_4 isozyme, on the immobilized oxamate gel column, was eluted by the column buffer containing no cofactor.

Fast evolution of B_4 isozyme

Antisera against the *P. esocinus* A_4 isozyme were elicited in mice by injecting the purified isozyme intraperitoneally. To find out cross-reactivity between anti-*P. esocinus* A_4 and the purified A_4 mixture of the antiserum and the A_4 isozyme was electrophoresed on polyacrylamide gel (Fig. 9). *P. esocinus* kidney A_4 isozyme was inhibited by anti-*P. esocinus* A_4 but *P. esocinus* B_4 isozyme was not, reflecting that the two homotetrameric LDH isozymes do not share common antigenic determinants.

It is highly accepted that the subunit A of vertebrate LDH has been relatively conservative in its evolution (Wilson et al., 1964; Yum et al., 1981). Expectedly, anti-*P. esocinus* A_4 also cross-reacts with the mouse A_4 isozyme (Fig. 10). These observations clearly indicate that the amino acid sequences of the antigenic determinants in *P. esocinus* B_4 isozyme has been experienced fast evolu-
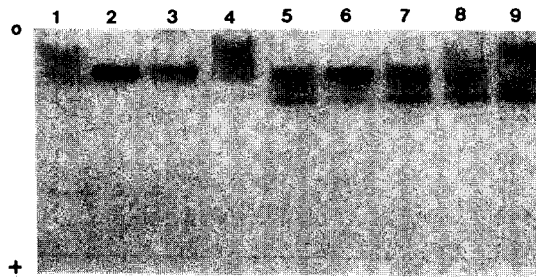
Fig. 9. Effect of anti-P. esocinus LDH A_4 upon the puri-
fied A_4 isozyme (A_4) and isozymes in P. esocinus kidney
extract (KE). 1, mouse antiserum (MS); 2, A_4; 3, A_4 +
MS (1:1); 4, A_4 + MS (1:3); 5, KE + MS (1:1); 6, KE;
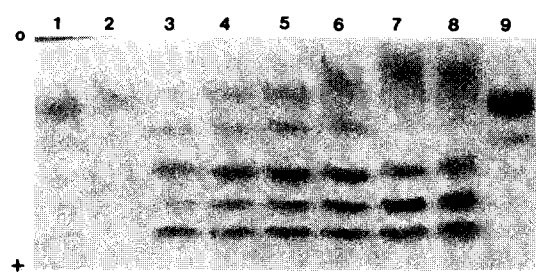7, KE + MS (1:2); 8, KE + MS (1:3); 9, KE + MS
(1:5).

Fig. 10. Effect of anti-P. esocinus LDH A_4 upon the
isozymes of mouse kidney extract. 1 and 9, mouse
skeletal muscle extract; 2, mouse antiserum (MS); 3,
mouse kidney extract (MKE); 4, MKE + MS (1:1); 5,
MKE + MS (1:2); 6, MKE + MS (1:3); 7, MKE + MS
(1:5); 8, MKE + MS (1:7).

tion than A_4 isozyme. The fast evolution of B_4
isozyme could be substantiated by following con-
siderations: (1) a higher frequency of electrophore-
tic variants is found at the gene for subunit B in
teleost (Markert and Falulhaber, 1965; Rainboth
and Whitt, 1974); (2) the subunits B in two Rana
populations are far more variable than the subun-
its A (Salthe, 1969) and (3) two alleles at the locus
producing subunit B are found in hemolysates of
certain human tribes (Boyer et al., 1963; Kraus
and Neely, 1964). Recently, by comparing the
amino acid sequences, Wistow et al. (1987) re-
ported that ε -crystallin, a prominent soluble pro-
tein of duck lenses, appeared to be identical to
duck LDH B_4 isozyme and suggested that ε -crys-
tallin is the result of a recent duplication of the
gene for the subunit B.

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Pseudogobio esocinus (Cypriniformes) 젖산수소이탈효소 동질사량체들의 정제, 역학 및 면역화학

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*Pseudogobio esocinus*의 심장, 신장 및 간 조직은 하부단위체 C를 함유하는 젖산수소이탈효소를 갖고 있음이 확인되었다. 하부단위체 A 및 B에 대한 유전자들의 조직 발현은 다른 포유동물의 것과 유사하였으며 분자량은 140,000 정도로 추정되었다.

Oxamate gel을 사용한 chromatography결과 A_4 동위효소는 NAD^+보다는 column buffer에 의해 용출되었다. B_4 동위효소는 CM-Sepharose column을 사용하여 부분 정제되었다. B_4 동위효소는 물론 A_4 동위효소도 고농도의 pyruvate에 의해 저해되었다. A_4 동위효소의 affinity chromatography 상 행동과 pyruvate 저해 정도로 보아 A_4 동위효소는 B_4 동위효소와 역학적으로 유사하다고 사료된다.

P. esocinus A_4 동위효소에 대한 항체는 mouse A_4 동위효소와 반응하지만 동종의 B_4 동위효소와는 반응하지 않는 특성으로 보아 하부단위체 B는 진화과정에서 보존성이 낮은 것으로 사료된다.