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MULTIPLE DEHYDROGENASES IN MARINE MOLLUSCS: ELECTROPHORETIC ANALYSIS OF ALANOPINE DEHYDROGENASE, STROMBINE DEHYDROGENASE, OCTOPINE DEHYDROGENASE AND LACTATE DEHYDROGENASE

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SUMMARY

Starch gel electrophoresis was used to analyze the number and activity of the dehydrogenases acting on pyruvate in various tissues of 14 species of marine molluscs (6 bivalves, 6 gastropods and 2 cephalopods). Four distinct enzymes, lactate dehydrogenase (LDH), octopine dehydrogenase (ODH), alanopine dehydrogenase (ADH: pyruvate + alanine + NADH ⇌ alanopine + NAD^+) and strombine dehydrogenase (SDH: pyruvate + glycine + NADH ⇌ strombine + NAD^+) were identified. All four enzymes were present in *Mytilus edulis* while various numbers and combinations were present in the other molluscs.

Key words: alanopine dehydrogenase, strombine dehydrogenase, octopine dehydrogenase, lactate dehydrogenase, molluscs, *Mytilus edulis*.

INTRODUCTION

The metabolic functions of intracellular free amino acids in molluscs are considerably more varied than those identified for vertebrate species. In molluscs, lactate dehydrogenase (LDH) and octopine dehydrogenase (ODH) are well known as terminal dehydrogenases for redox regulation in glycolysis. However, Fields (1976) noted that both LDH and ODH were absent from the oyster, *Crassostrea gigas*, and identified an enzyme which he termed 'alanopine dehydrogenase'. This enzyme catalyzed the formation of alanopine (*meso-N*-(carboxyethyl) alanine):

pyruvate + L-alanine + NADH ⇌ alanopine + NAD^+

and also the formation of strombine (*N*-(1-carboxymethyl)-D-alanine), when

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glycine replaced alanine as the amino acid substrate (Fields et al., 1980). Recently De Zwaan and Zurburg (1981) have shown that strombine can accumulate in the adductor muscle of *Mytilus edulis*.

In the present study we have examined the complement of 'pyruvate reductases' occurring in the tissues of a variety of marine molluscs, using electrophoretic separation to identify the enzymes present.

MATERIALS AND METHODS

Biochemicals were purchased from Sigma Chemical Co.

Animals

Mytilus edilus L., *Ostrea edulis* L., *Cerastoderma edule* (L.), and *Crepidula fornicata* (L.) were collected by dredge from the Tamar river estuary between 6 and 10 km from its mouth. *Patella aspera* Roding, *P. depressa* Pennant, *P. vulgata* L. and *Littorina littorea* (L.) were collected intertidally near Plymouth. *Glycymeris glycymeris* (L.), *Scaphander lignarius* (L.), *Pecten maximus* (L.), *Sepia officinalis* (L.), *Alloteuthis subulata* (Lamarck) and *Artica islandica* (L.) were collected offshore from Plymouth.

Extraction procedure

Tissues were homogenized (1:5 w/v) in 20 mM phosphate buffer, pH 7.2 using a Polytron homogenizer. For midgut glands or hepatopancreas, tissues were homogenized in 100 mM phosphate buffer, pH 7.2 containing 2 mM phenylmethylsulphonyl fluoride. The supernatant, after centrifugation at 30 000 *g* for 30 min, was used as the source of enzyme.

Electrophoresis

Vertical starch gel electrophoresis was run as described by Dando *et al.* (1979). Gels were run at 175–200 V using either a Tris-borate-EDTA buffer, pH 8.7 (for all species) or a *N*-(3-aminopropyl)-diethanolamine-citric acid buffer, pH 7.7 (to achieve better separation or to prevent loss of activity for *P. maximus*, *L. littorea*, *A. islandica*, *Cerastoderma edule* and *Crepidula fornicata* enzymes). Both buffers contained 20 μM NADH. All stains were applied as overlays containing 1.25% w/v agar, 100 mM triethanolamine-HCl, pH 7.0, and substrates: 0.1 mM NADH + 2 mM pyruvate (LDH) + 100 mM L-alanine (ADH) or 100 mM glycine (SDH) or 20 mM L-arginine (ODH). Other substrates, when tested, were added to the overlays at the following concentrations: 100 mM L-serine, 100 mM L-proline, 100 mM L-

cysteine, 100 mM $(NH_4)_2SO_4$, 50 mM L-valine, 20 mM L-threonine, 100 mM-methionine, 2 mM oxaloacetate, 2-oxobutyrate, or 2-oxoglutarate. As a control some gels were overlayed with NADH alone to detect NADH oxidase activity. Enzyme activity was detected by the reduction of fluorescence, due to NADH, under ultraviolet light.

RESULTS

Tissue distribution of enzyme activities

Tissue distributions, as determined by starch gel electrophoresis, are shown in Table I for LDH, ODH, alanopine dehydrogenase (ADH) and strombine dehydrogenase (SDH) from 14 species of marine molluscs. We have used a functional definition for assigning the names alanopine dehydrogenase or strombine dehydrogenase to the molluscan enzymes, due to the rather broad amino acid specificity of the dehydrogenases. We define alanopine dehydrogenase as the enzyme which has a high activity with alanine as substrate and shows a much lower activity with glycine. Strombine dehydrogenase, in contrast, utilizes glycine as its preferred amino acid substrate but also reacts readily with alanine. Preliminary experiments on the activities of the back reactions (strombine or alanopine + NAD^+) substantiate this definition. ADH from *M. edulis* reacts readily with alanopine as substrate but shows little activity with strombine, while SDH has a marked activity with either strombine or alanopine as substrate (Dando, 1981). In our nomenclature the adductor muscle 'alanopine dehydrogenase' of Fields et al. (1980) would, therefore, be considered to be a strombine dehydrogenase.

M. edulis contained all four dehydrogenases utilizing pyruvate: LDH, ADH, SDH and ODH. The other species lacked one or more of the enzymes. *Cerastoderma edule* lacked SDH, *P. maximus* and *Artica islandica* contained only SDH and ODH, *O. edulis* only ADH and SDH, *L. littorea* only ADH and LDH, *Crepidula fornicata* only SDH and LDH and *Alloteuthis subulata* and *Sepia officinalis* contained only LDH and ODH. The three *Patella* spp. and *Scaphander lignarius* contained only LDH while *G. glycmyeris* contained only ODH. The tissue distribution of each enzyme also varied from species to species (Table I). When both ADH and SDH were present (*M. edulis* and *O. edulis*), SDH predominated in the adductor muscle whilst ADH was present in all tissues. ODH, when present, had its highest activity in the adductor muscle and foot, or in the mantle of the cephalopods.

Electrophoresis revealed that LDH, ADH, SDH and ODH activities were catalyzed by distinct enzymes which were also distinct from glutamate dehydrogenase. Individual mussels, *M. edulis*, showed different banding patterns for ADH and SDH (Fig. 1) and for ODH. Each mussel showed activity in either one

TABLE I

Tissue distribution of the dehydrogenases LDH, ADH, SDH and ODH in 14 marine molluscs.

Species	Enzyme	Tissue				
		Adductor muscle	Mantle	Gill	Hepato-pancreas/ midgut gland	Foot
Mytilus edulis	LDH	tr	n.d.	n.d.	n.d.	tr
	ADH	tr	+ + +	+	+	+ + +
	SDH	+ + +	n.d.	n.d.	n.d.	n.d.
	ODH	+ + +	tr	tr	+	+ + +
Ostrea edulis	ADH	+ + (+ +)[a]	tr	+	+ + +	–
	SDH	+ + + (+)	n.d.	tr	tr	–
Cerastoderma edule	LDH	+ +	tr	tr	tr	+ +
	ADH	+ +	n.d.	n.d.	n.d.	+ + +
	ODH	+ + +	+	+	tr	+ + +
Glycymeris glycymeris	ODH	+ + +	tr	tr	–	+ + +
Pecten maximus	SDH	tr(tr)	tr	tr	tr	–
	ODH	+ + + (+)	+	tr	tr	–
Arctica islandica	SDH	+ + (+ +)	tr	tr	tr	+ + +
	ODH	+ + + (+ + +)	n.d.	n.d.	n.d.	+ +
Littorina littorea	LDH	+ +[b]	tr[c]	–	tr	+ +
	ADH	+ + +[b]	tr[c]	–	tr	+ +
Crepidula fornicata	LDH	–	tr	tr	tr	tr
	SDH	–	tr	tr	+	+ + +
Patella vulgata	LDH	–	+ + +	–	+ +	+ + +
P. aspera	LDH	–	+ + +	–	+ +	+ + +
P. depressa	LDH	–	+ + +	–	+ +	+ + +
Scaphander lignarius	LDH	–	+ + +	–	+	+ + +
						Brain
Alloteuthis subulata	LDH	–	tr	tr	–	tr
	ODH-A	–	tr	tr	–	+ +
	ODH-B	–	+ + +	+ +	–	n.d.
Sepia officinalis	LDH	–	tr	+	+	+
	ODH-A	–	n.d.	tr	+ +	+ +
	ODH-B	–	+ + +	tr	n.d.	n.d.

Starch gel electrophoresis and staining for enzymes was carried out as described in Materials and methods. If no data are given for an enzyme, it was not detected in any of the tissues. The relative enzyme activities within a species are indicated by the staining intensity: n.d. = not detectable, tr = trace, + = weak, + + = strong, + + + = very strong.

[a]Where the adductor muscle was divided into phasic and catch portions, data for the phasic portion are given first with those for the catch portion in parentheses.

[b]Data for columellar muscle.

[c]Mantle and gill tissues combined.

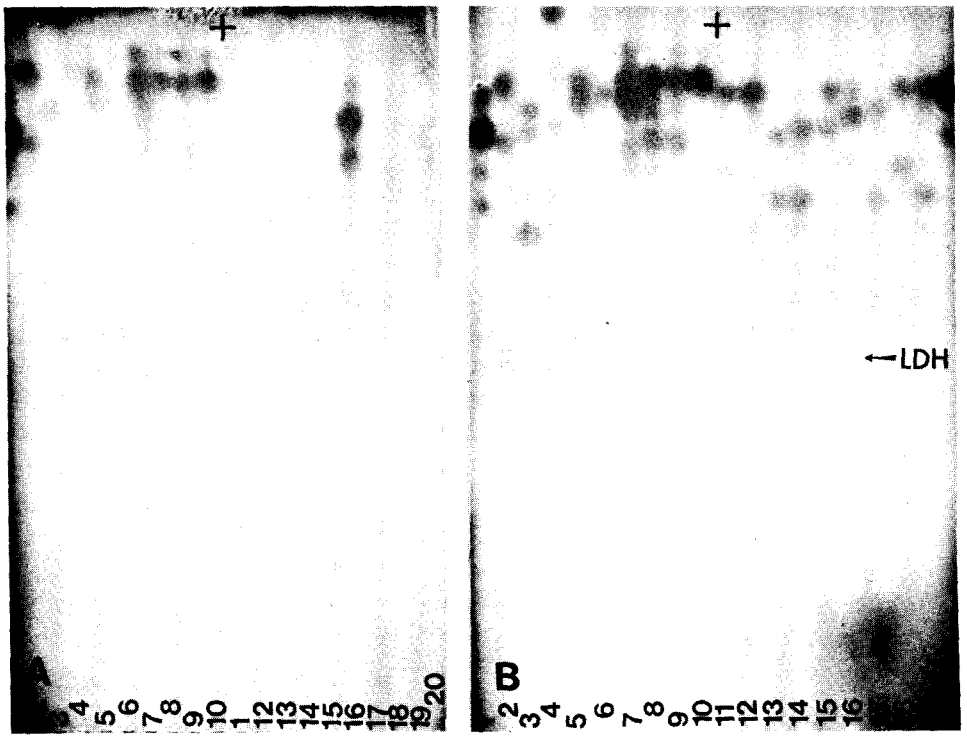

Fig. 1. Electrophoresis of *Mytilus edulis* posterior adductor muscle and foot extracts at pH 8.7. A, Stained with glycine + pyruvate + NADH to show SDH activity. B, Stained with alanine + pyruvate + NADH to show SDH + ADH activity. The gels were photographed under U.V. light. LDH is largely denatured at this pH and the position of the LDH zone is arrowed. ODH, when stained with either arginine + pyruvate + NADH, or with octopine + NAD, occurs below the LDH zone.
A and B, Slots 1–5, 7–10 and 16 contain extracts of posterior adductor muscle from *M. edulis* individuals 1–5, 7–10 and 6. Slots 6, 11–15 and 17–20 contain extracts of foot from *M. edulis* individuals 6, 1–5 and 7–10. Note in A the absence of SDH in foot and in B the presence of traces of ADH in adductor muscle.

or two major bands for each enzyme. For example, in Fig. 1 the adductor muscle extract from muscle 4 shows a fast migrating SDH band which stains with both glycine (A4) and alanine (B4) but which is absent from the corresponding foot extract (A14 and B14). With alanine, but not glycine, as amino acid substrate the foot extract stains for two ADH bands (B14) which also stain weakly in the adductor muscle extract (B4). Strongly staining extracts in Fig. 1 show weak 'satellite' bands, e.g. the two weak bands in A1: this phenomenon has previously been observed for ODH in *Cerastoderma edule* (Beaumont et al., 1980). Similar banding variation for ODH was found in *M. edulis*. Each extract stained for one or two major zones of activity which were always less anodal than the LDH, ADH or

SDH zones. Beaumont et al. (1980) have previously reported this ODH variation in samples of *M. edulis* adductor muscle and explained it in terms of co-dominant alleles at a single locus. ODH is known to be a monomer with a mol. wt. of 38 000 (Olomucki *et al.*, 1972). ADH and SDH from *M. edulis* also have mol. wts. of approx. 38 000 and are likewise probably monomers (Dando, 1981). The banding pattern for ADH or SDH is explicable if heterozygous individuals have two allozymes. Variation in any one of the four enzymes (ADH, SDH, ODH or LDH) in *M. edulis* did not coincide with the same variation in either of the other enzymes, implying that each is under separate genetic control. Similar individual variation was also found for SDH in *O. edulis* and for ADH in *Cerastoderma edule*, with animals displaying either one or two active bands.

Only ODH exhibited tissue-specific isoenzymes and these were confined to the two cephalopods, *Alloteuthis subulata* and *Sepia officinalis*. The brain-type isoenzyme, ODH-A, was the predominant form in brain and hepatopancreas and occurred in small amounts in other tissues. The mantle form of ODH, ODH-B, was the predominant form in the mantle muscles, gill and ventricle.

Substrate specificities of ADH and SDH

The substrate specificities of ADH and SDH were examined on starch gels by using different amino acids or keto acids in the staining overlay. The presence of polymorphic individuals of *M. edulis*, *O. edulis* and *Cerastoderma edule* enabled us to assign the substrate specificities unambiguously to the given enzymes in these species. ADH from *M. edulis* or *O. edulis* showed maximal staining with L-alanine as the amino acid substrate with trace amounts of staining in the presence of glycine. Of the other amino acid tested (L-cysteine, L-serine, L-threonine, L-methionine, L-proline, L-valine, L-arginine) and ammonia, ADH showed significant activity only with cysteine and serine. Low activities were found with methionine and threonine as the amino acid substrate for the enzyme from *M. edulis*. ADH from *L. littorea* reacted weakly when glycine replaced alanine, but not with any of the other amino acids tested. Three keto acids were tested: oxaloacetate, 2-oxobutyrate, and 2-oxoglutarate. ADH from *M. edulis* was equally active with either pyruvate or oxaloacetate. The reaction of ADH from *O. edulis* with oxaloacetate was obscured by strong malate dehydrogenase activity on the gel. Both of the above ADH enzymes showed a low activity in the presence of 2-oxobutyrate but none with 2-oxoglutarate as substrate.

SDH from all the molluscs tested stained well with both glycine and alanine as substrates. The enzyme from *M. edulis*, *O. edulis* and *A. islandica* also showed significant activity with either serine or cysteine. SDH from *M. edulis* reacted weakly with methionine and from *Crepidula fornicata* with serine > cysteine or threonine. The enzymes from *M. edulis* and *O. edulis* showed keto acid specificities in the order pyruvate ≥ oxaloacetate > 2-oxobutyrate. SDH from *P. maximus*

reacted more readily with 2-oxobutyrate than the *M. edulis* enzyme. Only SDH from *Crepidula fornicata*, which showed pronounced activities with pyruvate, oxaloacetate and oxobutyrate, was active when 2-oxoglutarate replaced pyruvate.

DISCUSSION

This study reveals a considerable diversity amongst marine molluscs in the number and type of 'pyruvate reductases' present in their tissues. Two distinct dehydrogenases acting on alanopine were found in *M. edulis* and *O. edulis*, one of which, strombine dehydrogenase, utilized strombine as the preferred substrate. This diversity of distribution and especially the presence of four enzymes in the posterior adductor muscle of *M. edulis* (LDH, ODH, ADH and SDH) is difficult to explain. ADH and SDH from the species examined here have quite broad substrate specificities. Both ADH and SDH utilize other amino acids (serine, cysteine and methionine) and keto acids (2-oxobutyrate and oxaloacetate) as substrates. Recently, substrate specificities for partially purified ADH and SDH from *M. edulis* have been reported by Dando (1981). These show good agreement with the specificities determined using gel overlays. It is likely that there are separate functions for the four enzymes in vivo and some of these will be discussed.

Glycolytic muscular work

The role of ODH as the terminal glycolytic dehydrogenase supporting 'burst' muscular work has been clearly demonstrated in a number of studies on molluscs (see review by Gäde, 1980). The observation that ODH occurs in highest activities in muscle and, when present, is frequently the dominant dehydrogenase (Table I) suggests that this enzyme may also be associated with glycolytic muscular work in the species studied. However, the absence of ODH (and also LDH) from the adductor muscle of *O. edulis* and other oysters (Fields, 1976) and the presence instead of SDH, implies that SDH is capable of acting in support of glycolytic muscular work. Similarly, the high activities of SDH in the foot of *A. islandica*, suggest that SDH could play a substantial role in glycolytic muscular activity. In *L. littorea*, this same function may be assumed by ADH (Table I).

Anaerobiosis

A second possible function for these dehydrogenases is in anaerobic metabolism and here ADH and SDH may play a more important role. Octopine is only a minor product of anaerobiosis and indeed Gäde (1980) has shown that the same muscle which accumulated octopine during muscular work, produced alanine and succinate under anaerobic conditions. Recently, concentrations of 3–5 mM strombine have

been found in the adductor muscle of *M. edulis* under anoxic conditions (De Zwaan and Zurburg, 1981). The data presented in this paper, which shows the occurrence of ADH and/or SDH in several of the well-studied, anoxia-tolerating intertidal invertebrates, indicate a clear capacity for these species to utilize the ADH or SDH reactions in anaerobic energy production.

Osmoregulation

Production of conjugated end products such as octopine, alanopine and strombine would minimize changes in tissue osmotic pressure during glycolysis and this could be of importance, particularly during long-term anaerobiosis in osmoconformers. The activity of the dehydrogenases is, obviously, closely linked to amino acid metabolism and their function could be linked to the regulation of intracellular free amino acid levels in some species.

In the presence of glutamate-pyruvate transaminase to remove an amino group from alanine, 2 molecules of alanine or 1 molecule of alanine plus 1 molecule of glycine could be interconvertible with single molecules of alanopine or strombine. A correlation exists between the occurrence of ADH and SDH and the known sizes of the intracellular glycine and alanine pools in several species. *Patella vulgata* and *G. glycymeris* have neither ADH nor SDH and are known to have small free amino acid pools with glycine and alanine concentrations of 2–8 mM (Gilles, 1972; Hoyaux et al., 1976). *L. littorea*, which contains ADH but no SDH, has alanine and proline as its major amino acids, with alanine showing the greatest reduction in concentration when the animal is acclimated to low salinities (Hoyaux et al., 1976). *Cerastoderma edule* contains SDH and has a large pool of both alanine and glycine (Shumway *et al.*, 1977). Both *O. edulis* and *M. edulis* contain SDH and high concentrations of glycine in the adductor muscles (Bricteux-Gregoire et al., 1964; Hoyaux et al., 1976). Other tissues of *M. edulis* contain only ADH. However, although the alanine:glycine ratio in these may be higher than in the adductor muscle it never exceeds unity (Zurburg and De Zwaan, 1981).

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