SERINE AND ALANINE RACEMASE ACTIVITY IN MARINE INVERTEBRATE TISSUES

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We have demonstrated previously that D-amino acids occur in the free amino acid pools in tissues of marine invertebrates in diverse phyla (Preston, R.L. Comp. Bioch. Physiol. 87B: 55-62, 1987). Furthermore, we showed that D-amino acids were transported and metabolized by invertebrate tissues (Preston, R.L. Comp. Bioch. Physiol. 87B: 63-71, 1987). The primary route of metabolism utilizes racemases, enzymes that interconvert D- and L-amino acids (Preston, R.L., et al., Bull. MDIBL 36: 86, 1997). Most of our earlier work focused on characterization and isolation of alanine racemase (see accompanying paper). In this paper we report the detection of alanine and serine racemases in representative marine invertebrates and demonstrate that the distribution of these two racemases may be independent of one another.

All animals used in these experiments were collected in Salsbury Cove, ME and maintained in running seawater aquaria until used. D-Amino acid content and racemase activity were measured using a coupled enzyme assay. Typical assay conditions to detect neutral D-amino acids were: tissue extract (50 µl), tetrasodium pyrophosphate (NaPP) buffer saturated with the chromophoric peroxidase substrate, *o*-dianisidine (50 mM NaPP, pH 8.5; 110 µl), D-amino acid oxidase (0.06 units; 20 µl) and horseradish peroxidase (0.02 mg; 20 µl) and water, 50 µl. The reaction was run at room temperature in a 96 well microplate and the product formation (oxidized *o*-dianisidine) measured at 490 nm using an ELISA plate reader. The absorbance was read every 10 min initially and at longer intervals for up to 8 hours. The racemization reaction proceeds slowly and the rates of reaction were usually calculated from the slopes of time courses plotted from these data. For all measurements there were 4 to 8 replicates depending on the condition. A variety of positive (containing added D-amino acid) and negative controls were also included. The racemase assay used a similar procedure except that 200 mM L-alanine or 200 mM L-serine was added to the extract and the formation of D-amino acid measured. This assay can detect neutral D-amino acids at concentrations from 50 µM or higher.

Table 1 shows the typical results from analysis of D-amino acid content and serine and alanine racemase activity in three marine invertebrate species. For screening purposes, small whole animals or the combined viscera (*Limulus*) were used to prepare extracts. The "activity Ratio" shown in Table 1, [A_{490} each condition/A_{490} (extract only control)], compares the A_{490} of the control (extract only) condition to endogenous D-amino acid or racemase activity. A "Ratio" significantly >1.0 indicates the presence D-amino acid or racemase activity.

In the moon snail, *Lunatia heros*, vigorous alanine racemase activity was detected (activity Ratio = 4.80), but no significant endogenous D-amino acid content or serine racemase activity was present. Rapid alanine racemization is not necessarily inconsistent with the apparent absence of endogenous D-amino acids because the limit of detection of the coupled enzyme assay is about 50 µM and the

concentrations present in *Lunatia* may be below this limit. Furthermore, even if large amounts of D-alanine are produced, it may be further metabolized, released or sequestered from the extractable free amino acid pool. In the visceral tissues of the horseshoe crab, *Limulus polyphemus*, significant serine racemase activity was detected (activity Ratio = 2.93), but with no apparent alanine racemase activity or significant concentrations of neutral D-amino acids. In the starfish, *Asterias forbesi*, endogenous D-amino acids were detected (activity Ratio = 1.81) as well as both alanine racemase (activity Ratio = 2.52) and serine racemase (activity Ratio = 3.85).

Table 1: Serine and alanine racemase activities in marine invertebrate tissue extracts*.

Species	*Lunatia heros* whole			*Limulus polyphemus* viscera			*Asterias forbesi* whole		
	A_{490}/hr**	SE	Ratio***	A_{490}/hr**	SE	Ratio***	A_{490}/hr**	SE	Ratio***
Extr. Only	0.017	0.001		0.021	0.001		0.032	0.001	
Total D-AA	0.019	0.001	**1.13**	0.024	0.002	**1.12**	0.058	0.004	**1.81**=
Ala Racemase	0.080	0.002	**4.80**=	0.025	0.001	**1.19**	0.081	0.005	**2.52**=
Ser Racemase	0.018	0.001	**1.10**	0.062	0.002	**2.93**=	0.123	0.005	**3.85**=

* See text above for explanation of the coupled enzyme assay. All samples contained NaPP buffer pH 8.5 with saturating concentrations of the chromophoric reagent, *o*-dianisidine. Abbreviations: Extr. Only = tissue extract in NaPP buffer; Total D-AA = endogenous D-AA measured under conditions that do not stimulate racemase activity; Ala Racemase. = tissue extract plus 200 mM L-alanine; Ser Racemase. = tissue extract plus 200 mM L-serine.

** The absorbance at 490 nm was read against a NaPP blank for 176 to 236 min incubation period and the rate (A_{490}/hr) was calculated. Note: The symbol (=) indicates that a statistical comparison (t-test) of the absorbance values for each demarcated experimental condition compared with the respective control conditions (extract only) are significantly different from the control at the $p < 0.05$ or better ($n = 4$ or 8).

*** Ratio = [A_{490} each condition/A_{490} (extract only control)]. The denominator compensates for the intrinsic color of the extract and the assay enzymes. An activity Ratio significantly >1.0 indicates the presence D-amino acid or racemase activity.

As far as we are aware this is the first report of serine racemase activity in marine invertebrate tissues although serine racemase has been identified and cloned from mouse brain (Wolosker et al., PNAS 96: 13409-13414, 1999). Furthermore, these data demonstrate that racemase activity for alanine and serine appears to be independently distributed depending on species. Thus, alanine racemase appears to be present only in *Lunatia heros* and serine racemase activity appears to be expressed only in *Limulus polyphemus*. However, in *Asterias forbesi* both activities occur together. It is perhaps not particularly surprising that each amino acid appears to have a specific selective racemase but it is theoretically possible that a single racemase with broad specificity could mediate racemization for all common amino acids. The results reported here are consistent with those for bacterial racemases that are highly selective (Adams, E., Adv. Enzymol. Relat. Areas Mol. Biol. 44: 69-138, 1976). These data are also consistent with the limited biological and genetic information available on eukaryotic racemases (serine racemase from mouse brain, Wolosker et al., PNAS 96: 13409-13414, 1999; alanine racemase from plant fungus, *Cochliobolus carbonum,* Cheng and Walton, J. Biol. Chem. 275: 4906-11, 2000). We are extending these observations using biochemical and molecular approaches to identify the varieties of racemases in marine invertebrate tissues. (Valerie Petit was a Hancock County Scholar funded in part by the SETH Program).