RACEMASE ACTIVITIES IN THE TISSUES OF MARINE MOLLUSKS

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We have shown 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) and are transported and metabolized by invertebrate tissues (Preston, R.L. Comp. Bioch. Physiol. 87B: 63-71, 1987). The principal metabolic pathway interconverts D- and L-amino acids via equilibrative enzymes called racemases (Preston, R.L., et al., Bull. MDIBL 36: 86, 1997). We focused a substantial portion of our previous studies on the biochemical and molecular characterization of alanine racemase which commonly occurs in many species (Preston, R.L., et al., Bull. MDIBL 36: 86, 1997; Preston, R.L., et al., Bull. MDIBL 40: 116, 2001). In this paper we report the detection of a putative threonine racemase for the first time and as well as identifying the presence of alanine and serine racemases in three species of mollusk.

Animals used in these experiments were collected in Salsbury Cove, ME and maintained in running seawater aquaria. D-Amino acid content and racemase activity were measured using a coupled enzyme assay. Standard assay conditions used 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 (*o*-dianisidine dimer) measured at 490 nm using an ELISA plate reader (Bio-Tek Instruments, Winooski, VT). A variety of positive (containing added D-amino acid) and negative controls were also included. The absorbance was read every 15 min initially and at longer intervals for up to 10 hours. For all measurements 4 to 8 replicates were done. The racemase assay used a similar procedure except that 200 mM L-threonine, L-alanine or L-serine was added to the extract. This assay can detect neutral D-amino acids at concentrations from 50 µM or higher but the detection of racemases depends on stimulating D-amino acid formation from L-amino acids in excess of that detected in initial baseline measurements.

Table 1 shows the results from analysis of D-amino acid content and serine, threonine and alanine racemase activity in three species of mollusks. 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 slipper shell, *Crepidula fornicata*, both high concentrations of neutral D-amino acids (activity Ratio = 4.85) and vigorous alanine racemase activity was detected (activity Ratio = 14.6). It is usually the case that high D-amino acid content and vigorous racemase activity occur together but this is not always the case since the limit of detection of the coupled enzyme assay is about 50 µM and the concentrations present in some species may be below this limit (Preston,

R.L., et al., Bull. MDIBL 40: 116, 2001). In the foot muscle of Stimpson's whelk, *Colus stimpsoni*, significant D-amino acid content (activity Ratio = 1.81), and high serine racemase activity (activity Ratio = 5.74) was detected. In the gill tissue of the blue mussel, *Mytilus edulis*, D-amino acids were detected (activity Ratio = 4.5) as well as threonine racemase (activity Ratio = 8.25).

Table 1:
Racemase activities in marine invertebrate tissue extracts*.

Species	*Cepidula fornicata* Whole			*Colus stimpsoni* foot muscle			*Mytilus edulis* gill		
	A_{490}/5hr**	SE	Ratio***	A_{490}/5hr**	SE	Ratio***	A_{490}/5hr**	SE	Ratio***
Extr. Only	0.041	0.002		0.031	0.001	–	0.008	0.001	–
Total D-AA	0.199	0.008	**4.85**$^{++}$	0.056	0.002	**1.81**$^{++}$	0.038	0.001	**4.5**$^{++}$
Ala Racemase	0.600	0.014	**14.6**$^{++}$	–	–	–	–	–	–
Ser Racemase	–	–	–	0.178	0.008	**5.74**$^{++}$	–	–	–
Thr Racemase	–	–	–	–	–	–	0.066	0.002	**8.25**$^{++}$

* 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; Thr Racemase. = tissue extract plus 200 mM L-threonine. A small amount of D-amino acid contamination is sometimes present in some commercially available L-amino acid reagents. Racemase measurements were corrected for this by subtraction of the mean of control values for wells containing only L-alanine, serine or threonine, as appropriate..

** The absorbance at 490 nm was read against a NaPP blank for 197 to 591 min incubation period and the rate (A_{490}/5hr) was calculated. Long incubations are typically required for accurate detection of racemase activity and the 5 hr time period was a typical experimental time required.

*** 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. 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).

As far as we are aware this is the first report of threonine racemase activity in marine invertebrate tissues and in eukaryotic tissues. Alanine racemase is quite widely distributed in marine invertebrate tissues and only recently has serine racemase been detected in these organisms as well (Preston, R.L., et al., Bull. MDIBL 40: 117-118, 2001). Since threonine is a close structural analogue of serine, caution should be exercised in assuming this is a racemase different from the serine racemase, although our preliminary data suggests it is different. These data are also consistent with the fact that each amino acid appears to have a specific selective racemase as is the case in bacteria where racemases have been quite well characterized (Adams, E., Adv. Enzymol. Relat. Areas Mol. Biol. 44: 69-138, 1976). We are in the process of molecular characterization of the alanine racemase from *Mya arenaria* and plan to extend this sort of analysis to other racemases (Preston, R.L., et al., Bull. MDIBL 40: 116, 2001). (Amanda Bryant was an NSF-REU Fellow (DBI-9820400); Ishani Sud and Velvet Williams were Burroughs-Wellcome Fellows; Alexandra Budhai was a Hancock County Scholar funded in part by the SETH Program).