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Amylase Polymorphism of *Littorina brevicula* from Polluted and Unpolluted Sites, Korea

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The toxic effects of heavy metals on organisms have received considerable scientific interest in recent years. Heavy metal ions are waste products of many industrial processes and be released into the ocean, causing environmental pollution. Environmental physiological stresses allow us to subject a polymorphic population which would be easily affected by these stresses, and determine the differential fitness of allozyme genotypes in the field. A lot of evidence drawn from both natural and laboratory populations indicate that the distribution of allozyme polymorphism is nonrandom and varies in spatio-temporal environmental variation (Bryant 1974; Nelson and Hedgecock 1980). In laboratories, the effects of pollution on the genetic structure of marine organisms has been studied. It has been reported that the marine animals selected the specific genotypes on the effect of heavy metal (de Nicola *et al.* 1993; Benton *et al.* 1992 a, b; Lavie and Nevo 1982, 1986; Ben-Shlomo and Nevo 1988; Nevo *et al.* 1981). These experiments have shown both tolerance and sensitivity of allozyme genotypes to the pollutants. They have also shown that heavy metals affect the enzyme functions and the sex ratio of the species. Unfortunately, most of the studies were performed to determine the effects of pollutants on marine organisms in the laboratory, and they did not determine allozyme variance of natural population in the field.

Studies were performed on heavy metal pollution of sea water, sediments, oyster, mussels and *Littorina brevicula* (Philippi) in the coastal environment of Korea from 1995 to 1998 and we found that heavy metal (especially Cd, Zn, Cu and Pb) is accumulated in the digestive gland, intestine and gill of *L. brevicula* (Lee ***et al.*** 1996; Song *et al.* 1997). We also investigated the status of marine pollution in Korea, using the combined k-dominance curve for species biomass and individual numbers. The macrozoobenthic community structures of polluted sites were changing and the species richness composition was lower in polluted sites than in unpolluted sites (Rho *et al.* 1997). These results of Dokdong, Chundo and Daejung-chun showed greater impact than the results of Isudo, Tangsa and Jin-ha. The periwinkle *Littorina brevicula* has been used widely for the assessment of heavy metal pollution in the marine environment. This periwinkle is partly exposed to air and must be more tolerant to a wide range of heavy metals, temperatures, salinity, oxygen concentration and other factors affected by tidal changes (Reid 1996). Also, many polymorphic loci and significant variation in allozyme heterozygosity was found in *L. brevicula* (Tatarenkov 1992). Amylase acts primarily in a stomach which contains contaminated organic particles that are able to bind to metal ions. Mizrahi and Arhituv (1989) found the effects of different concentrations of heavy metals (Cd, Hg, Zn, Cu and Pb) on the activity of lactate dehydrogenase, malate dehydrogenase and cytochrom oxidase but not amylase in *D. trunculus.* Yan *et al.* (1996) have observed the inhibition of metals (Pb, Cd, Zn and Hg) on the activity of a -amylase from the digestive gland of the green mussel, *Pernu viridis.* They showed that

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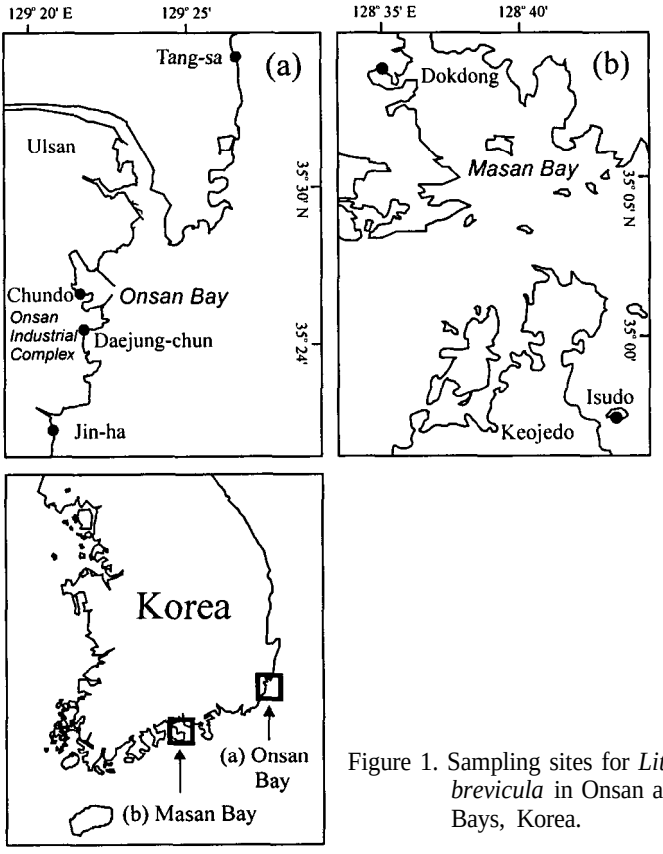

Figure 1. Sampling sites for *Littorina brevicula* in Onsan and Masan Bays, Korea.

Hg was the most effective inhibitor of amylase, followed by Zn, Cd and Pb in this order. according to Km and Vmax values. The bioaccumulation of heavy metals in the digestive gland and intestine causes the inhibition of digestive enzymes, which will lead to a lower growth rate of green mussels.

Thus, to compare the effects of heavy metal (Cd, Zn, Cu and Pb) pollution, this present work determined the amylase allozyme variation, total wet weight, sex ratio, penis size and the number of penial glands of natural *L. brevicula* populations in polluted and unpolluted sites.

MATERIALS AND METHODS

One thousand five hundred and one individuals of *L. brevicula* were collected from the polluted sites (Dokdong, Chundo, and Daejung-chun) and from the unpolluted sites (Isudo, Tangsa and Jin-ha) of Masan and Onsan Bays in Korea (Figure 1). The polluted sites were identified based on the degree of heavy metal concentrations in our previous works (Lee et al. 1996; Song et al. 1997; Rho et al. 1997). The specimens were collected in February and July of 1996 and 1997. The shell height of the collected *L. brevicula* ranges from 5mm to 15mm. The samples were transported on dry ice and kept at -70% until analysis. The digestive glands were excised from the *L. brevicula* and homogenized in Phosphate-buffered saline of the same volume, then centrifuged at 5000 x g for 10 min at 4°C. The supernatant was used for electrophoresis. Electrophoresis was carried out with a 0.19M Tris-HCl buffer (pH 8.8) for the 1.2% (w/v) horizontal agarose gel, and a 0.3M Boric acid-NaOH buffer (pH 8.0) for the electrode. To develop the gel,

it was incubated at 37°C in 0.9% soluble starch containing 0.2% calcium chloride. Then it was immersed in 1% acetic acid and was dyed with KI-iodine solution. The X^2-test was used to compare findings for polluted sites with those for unpolluted sites.

For the investigation of sex ratio, number of penial gland and variation of the penis under the right cephalic tentacle, the organisms which had been thawed were observed under the stereomicroscope.

RESULTS AND DISCUSSION

The amylase isozyme genotypes of *L. brevicula* were observed, and the 4 alleles found *Amy*1* (Rf 0.49), *Amy*2* (Rf 0.42) *Amy*3* (Rf 0.32) and *Amy*4* (Rf 0.27). Amylase isozyme was monomeric. The frequency of the amylase genotypes and alleles is shown in Table 1. *Amy*2* and *Amy*3* alleles appeared more often, as their frequencies were 0.32 and 0.62 respectively. The frequency of the *Amy*3/Amy*3* amylase genotype of the population in the polluted sites was significantly higher than that of the unpolluted sites, in both Masan and Onsan Bays ($p<0.01$). However, the frequency of *Amy*2/Amy*2* and *Amy*2/Amy*3* ($p<0.05$) in the polluted sites were lower than those of the unpolluted sites, in both Masan and Onsan Bays. *Amy*3/Amy*3* is considered to be related to tolerance, but *Amy*2/Amy*2* and *Amy*2/Amy*3* to sensitivity. This is similar to the work of Lavie and Nevo (1986) in which genetic selection of homozygotes occurred by aspartic acid

Table 1. Amylase polymorphism and allele frequencies of *L. brevicula* in polluted and unpolluted sites

Sites	N	Amylase genotypes										Allele frequencies			
		*Amy*1/Amy*1* N (%)	*Amy*2/Amy*2* N (%)	*Amy*3/Amy*3* N (%)	*Amy*4/Amy*4* N (%)	*Amy*1/Amy*2* N (%)	*Amy*1/Amy*3* N (%)	*Amy*1/Amy*4* N (%)	*Amy*2/Amy*3* N (%)	*Amy*2/Amy*4* N (%)	*Amy*3/Amy*4* N (%)	*Amy*1*	*Amy*2*	*Amy*3*	*Amy*4*
Masan Bay															
Dokdong+	226	1 (0.5)	24 (10.6)	145[1] (64.1)	8 (3.5)	0 (0.0)	2 (0.9)	0 (0.0)	31[2] (13.8)	8 (3.5)	7 (3.1)	0.01	0.19	0.73	0.07
Isudo	196	3 (1.5)	27 (13.8)	101 (51.6)	3 (1.5)	0 (0.0)	1 (0.5)	0 (0.0)	52 (26.5)	3 (1.5)	6 (3.1)	0.02	0.28	0.66	0.04
Onsan Bay															
Chundo+	270	2 (0.7)	34 (12.6)	114[1] (42.2)	6 (2.2)	2 (0.7)	3 (1.1)	1 (0.4)	96[2] (35.6)	4 (1.5)	8 (3.0)	0.02	0.31	0.62	0.05
Tangsa	339	12 (3.5)	61 (18.0)	89 (26.3)	2 (0.6)	3 (0.9)	6 (1.8)	1 (0.3)	160 (47.2)	4 (1.2)	1 (0.3)	0.05	0.43	0.51	0.01
Daejung -chun+	256	0 (0.0)	18 (7.0)	114[1] (44.5)	1 (0.4)	3 (1.2)	0 (0.0)	0 (0.0)	111[2] (43.3)	0 (0.0)	9 (3.5)	0.01	0.29	0.68	0.02
Jin-ha	214	6 (2.8)	30 (14.0)	50 (23.4)	0 (0.0)	7 (3.3)	3 (1.4)	0 (0.0)	111 (51.9)	0 (0.0)	7 (3.3)	0.05	0.41	0.52	0.02
Total	1501	24 (1.6)	194 (12.9)	613 (40.8)	20 (1.4)	15 (1.0)	15 (1.0)	2 (0.1)	561 (37.4)	19 (1.3)	38 (2.5)	0.03	0.32	0.62	0.03

+ ; polluted site

1. $p<0.01$; polluted vs unpolluted sites of *Amy*3/Amy*3*

2. $p<0.05$; polluted vs unpolluted sites of *Amy*2/Amy*3*

transaminase on marine gastropod ***C. sacbridum*** exposed to cadmium and mercury. The resistance for an individual organism may depend on genetic differences, which is confirmed by the fact that pollutants act by selecting some PGM genotypes (de Nicola et al. 1993; Ben-Shlomo and Nevo 1988). If the changes in allozyme frequencies of organisms are indeed sensitive and adapted variantly according to the level and type of the pollutant, such genetic changes of resistance to toxicity can be used as a promising biological indicator of pollution (Nevo et al. 1981; Baker et al. 1985; Benton and Guttman 1992a, 1992b).

Heterozygotes from the polluted sites in Masan and Onsan Bays were significantly less than those of the unpolluted sites. The frequency of heterozygotes of organisms in Onsan Bay (0.508) was higher than that of Masan Bay (0.264) (Table 2). This was supposedly caused by a geographical difference. The polluted site of Masan Bay is in a semiclosed sea and polluted by sewage and industrial wastes, but the sites of Onsan Bay are in an exposed sea and polluted by petrochemical wastes and heavy metals. These results indicate that the *L. brevicula* population of Onsan Bay has indeed more heterozygotes than that of Masan Bay. The amylase genotype variance of the natural *L. brevicula* population showed no seasonal differences. There were no differences of amylase band intensity of *L. brevicula* between polluted and unpolluted sites.

Table 2. Heterozygote and homozygote ratios in polluted sites

Sites	Types	Polluted sites		Unpolluted sites		p
		Frequency	Heterozygote /Homozygote	Frequency	Heterozygote /Homozygote	
Onsan Bay	Homozygote	0.549	0.820	0.436	1.290	<0.005
	Heterozygote	0.451		0.564		
Masan Bay	Homozygote	0.788	0.270	0.684	0.462	<0.01
	Heterozygote	0.212		0.316		

P value; polluted vs unpolluted sites of the heterozygote/homozygote ratio

The total wet weight of *L. brevicula* varied from 290 to 1036 mg in the collecting sites. Ecotypic variation of the total wet weight of *L. brevicula* was obseved among sampling sites regardless of pollution. The barnacle ecotypes which appeared in Dokdong site's study were very small because the site was wave-exposed and rocky. Ecotypic variation of shell sculpture is particularly well developed in *Littorina* species. The marine gastropod *Partella caerulea* showed a difference in shell length regardless of a differential mercury concentration (0.09-1.0 μg/g d.w) (Raviv and Krumgalz 1981). However, the body length of marine Isopoda *Idotea bultica* was shortened by a different cadmium concentration (650 μg/g d.w) (de Nicola et al. 1993). With these results being considered, when marine populations were exposed to the extreme quantity of heavy metals, the body length and total wet weight of the marine organisms seemed to have been affected. However, because the Korean coast is not heavily polluted with heavy metals, the total wet weight of *L. brevicula* was not different between polluted and unpolluted sites. There was a difference in sex ratio between polluted and unpolluted

sites (Table 3). Males were reduced in the polluted sites, as the female to male ratio in the polluted sites (1.79:1) was higher than in the unpolluted sites(l.57:1). Similarly, the frequency of males of marine Isopoda *Idotea baltica* exposed to a high cadmium concentration was decreased (de Nicola et al. 1993). These results showed that pollutants cause a dramatic shift in the sex ratios of the population. It was suggested that sex ratio change might be associated with pollution. The penis of *L. brevicula* varied in size and in the number of mamilliform glands. The number of glands extended from 0 to 9, whereas the usual number is 5-7 glands. Furthermore, small penis were present in

Table 3. Sex ratio of *L. brevicula* in polluted and unpolluted sites

	Polluted sites			Unpolluted sites			
	Dokdong^{++}	Chundo^{+}	Daejung-chun^{+}	Isudo^{++}	Tangsa^{+}	Jin-ha^{+}	Total
F	99	77	70	56	85	89	476
M	55	42	40	33	54	61	285
Total	154	119	110	89	139	150	761
Sex ratio (F/M)	1.80	1.83	1.75	1.69	1.57	1.45	1.68

+ Onsan Bay
++ Masan Bay

polluted sites more than in unpolluted sites. It could therefore be considered that penis variation was related to pollution of heavy metals. The presence of a penis is normally diagnostic of males, but the development of a small penis has occasionally been recorded as an abnormality in females. Such psedohermaphroditism is sometimes a response to the anti-fouling pollutant tributyltin. However, Reid (1996) reported that the mamilliform penial glands of *L. brevicula* are 0-7. Seasonal variation is common in *Littorina,* as *L. brevicula* shed their penis in May to June following breeding (Kizaki 1986).

As a consequence, pollution by heavy metals (Cd, Zn, Cu and Pb) in marine environments might be responsible for the amylase polymorphism, sex ratio and penis type of the natural *L. brevicula* population. The genetic selection of amylase genotype in *L. brevicula,* especially homozygotes, can possibly be used as a monitoring system for marine pollutants.

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