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Research paper

# Protein diagenesis in *Patella* shells: Implications for amino acid racemisation dating

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ABSTRACT

The inter- and intra-crystalline fractions of *Patella vulgata* limpets recovered from archaeological sites in Northern Spain (covering Neolithic, Mesolithic, Magdalenian, Solutrean, and Aurignacian periods) were examined for amino acid composition and racemisation over time. The calcitic apex and rim areas of the shells were found to probably be composed of similar proteins, as the D/L values and amino acids were comparable and varied in the same way with increasing age; however, the mineral structures present in these areas differed. The aragonitic intermediate part of the shell showed a distinctly different amino acid composition and mineral structure. The main protein leaching from the inter-crystalline fraction occurred within the first 6000 yr after the death of the organism. In contrast, the intra-crystalline fraction — comprised of a different protein composition than the inter-crystalline fraction — appeared to behave as a closed system for at least 34 ka, as reflected by the lack of a significant decrease in the amino acid content; however, changes in the amino acid percentages occurred during this period. The concentration of aspartic acid remained almost constant with age both in inter- and intra-crystalline proteins, and its contribution to the total amino acid content increased with age at the expense of other amino acids such as glutamic acid, serine, glycine and alanine. Temperature is thought to play a key role in the amino acid racemisation of *P. vulgata* and could explain why in the localities belonging to the Gravettian and Solutrean period, which formed during relatively cold conditions, D/L values were similar to those detected in shells from sites formed during the Magdalenian.



## 1. Introduction

The first attempts to establish the chronology of shell middens using amino acid racemisation/epimerisation date back to the 1970s when Masters and Bada (1977) and Wehmiller (1977) analysed marine bivalve molluscs (*Chione*) from California. Several studies have demonstrated that amino acid racemisation (AAR) is a satisfactory tool for dating palaeontological and archaeological sites, including the use of limpets recovered from Palaeolithic and Mesolithic anthropogenic shell middens (Bateman et al., 2008; Ortiz et al., 2009a; Demarchi et al., 2011). Shell middens often accumulate relatively rapidly but they are subject to complex taphonomy. Consequently, large sample sizes for dating are required to resolve issues of intra-site chronology (e.g. Glover et al., 1990; Stein and Deo, 2003). The number of samples commonly used for the age calculation of a single level through AAR allows not only the rejection of anomalous results, but also an understanding of time-averaging and the time over which a certain site formed.

Some uncertainties regarding the protein diagenesis of limpet shells remain. Further research is therefore required to clarify the processes of protein preservation and degradation and the concomitant success of AAR for dating archaeological localities. Recent studies of modern limpets performed by Demarchi et al. (2011, 2013a,b) showed the potential of intra-crystalline proteins in *Patella* shells for AAR geochronology. In these studies, artificial diagenesis was induced in proteins (both inter- and intra-crystalline protein fractions, and the isolated intra-crystalline fraction) of modern *Patella* shells through high-temperature experiments (80°, 110°, and 140 °C) over a range of times (0–5738 h). The protein breakdown was quantified by measuring the extent

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and racemisation of various amino acids. This provided data on protein diagenesis in modern limpets; however, it is pertinent to reveal the circumstances of protein degradation in fossil representatives. In this regard, here we examined the amino acid content and D/L values in limpets (*Patella vulgata*) collected from several archaeological sites of known ages (dated by $^{14}C$) covering the Aurignacian (ca. 34 cal. ka BP), Gravettian (ca. 27.5 cal. ka BP), Solutrean (ca. 26.5–20.5 ka cal. BP), Lower, Middle and Upper Magdalenian (20.5–12.0 cal. ka BP), Azilian (ca. 12.0–10.8 cal. ka BP), Mesolithic-Asturian (10.8–6.3 cal. ka BP), and Neolithic (ca. 6.3–5.8 cal. ka BP) periods. *P. vulgata* was chosen because this limpet is the most common species in shell middens in Northern Spain (González-Morales, 1982; Bailey and Craighead, 2003; Gutiérrez-Zugasti, 2009, 2011; Álvarez-Fernández, 2011). We examined the behaviour of the whole protein content (inter- and intra-crystalline proteins) and the intra-crystalline fraction separately, the latter by bleaching prior to analysis.

Several studies (Haugen and Sejrup, 1992; Wehmiller, 1993; Torres et al., 1999) have reported intra-shell variation of D/L values depending on the part of the carapace from which the sample is recovered. We therefore also studied the amino acid content and D/L values of two parts of the shell (apex and rim) in samples of various ages.

## 2. Material and methods

The samples were collected from 12 sites in the regions of Asturias and Cantabria (Northern Spain) previously excavated in archaeological campaigns (Fig. 1). Permission was obtained for sampling the limpets. Once collected, the shells were stored at the "Museo Arqueológico de Asturias", the "Museo de Prehistoria y Arqueología de Cantabria", and the "Museo y Centro de Investigación de Altamira". Limpets were cleaned with water after their collection and stored in boxes at room temperature (15 °C) in the museums. The coordinates of the localities are reported in Table 1 (Fig. 1), together with the time period of the archaeological level sampled.

*P. vulgata* shells from the levels belonging to the Upper Palaeolithic (Aurignacian, Gravettian, Solutrean, Magdalenian, Azilian), Mesolithic (Asturian) and Neolithic (Table 1) periods were analysed for AAR. For comparitive purposes, modern specimens were recovered from Cue beach (Asturias), located close to the archaeological localities (Fig. 1).

### 2.1. Petrographic analysis

Selected *P. vulgata* shells from modern individuals were cut into thin sections along their major axis and placed on microscope slides. To determine the distribution of minerals and the organic matrix, the sections were submerged in Feigl's and Mutvei's solutions for 5 min and observed under a Nikon microscope.

To distinguish between the two calcium carbonate polymorphs that mollusc shells generally form, we applied Feigl's solution, which was prepared following Feigl (1937, in Friedman, 1959). This procedure stained aragonite crystals black, while calcite ones remain unstained.

To highlight the organisation of the organic matrix and the crystal arrangement, we used Mutvei's solution (Mutvei et al., 1994), following the modifications described by Schöne et al. (2005): one litre of Mutvei's solution consists of 500 ml 1% acetic acid, 500 ml 25% glutaraldehyde and ca. 5–10 g Alcian blue powder. The use of Mutvei's solution facilitates the identification of micro-growth structures in carbonates of biogenic origin by staining organic matrix laminae and crystal envelopes in shades of blue.

### 2.2. Amino acid racemisation

Between 4 and 11 *P. vulgata* shells (analytical samples) from each archaeological level were analysed for amino acids. The use of monospecific or monogeneric samples reduces taxonomically-controlled variability in D/L values (Murray-Wallace, 1995; Murray-Wallace and Goede, 1995). In the laboratory, shells were

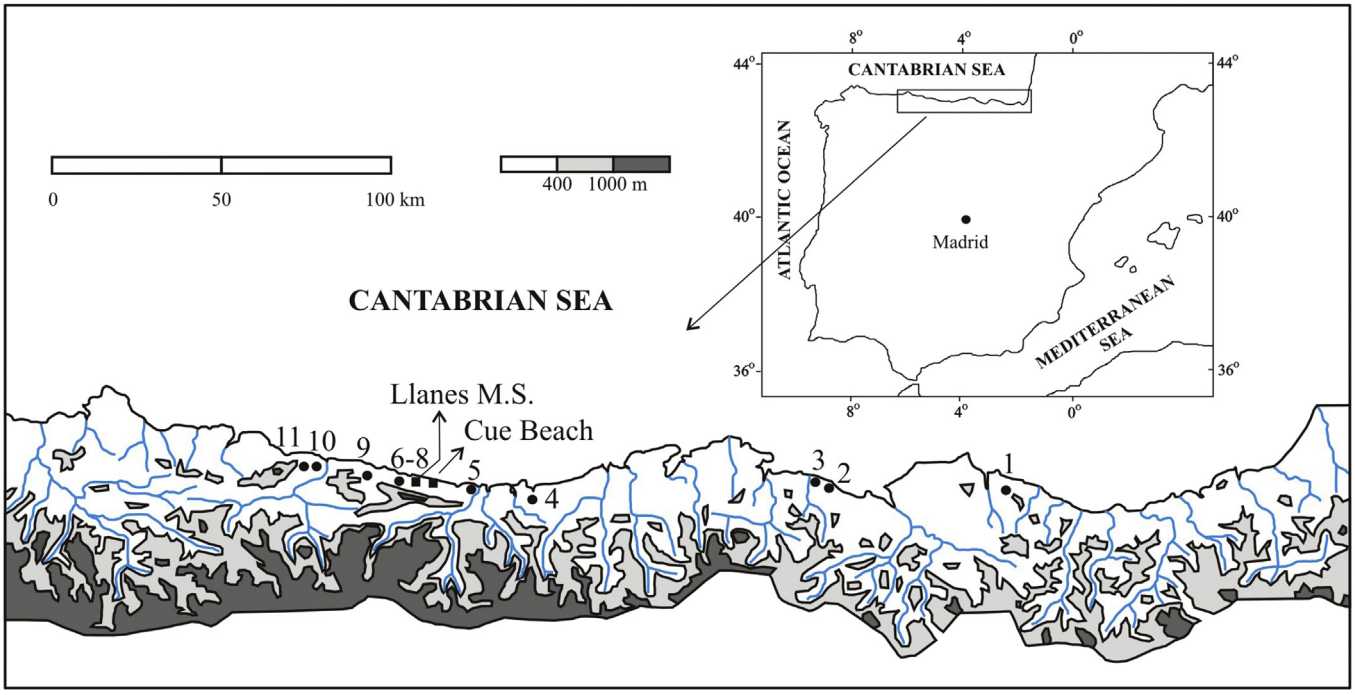


**Fig. 1.** Geographical location of the caves studied. 1-Kobaederra, 2-El Cuco, 3-Arenillas, 4-Fuente Salín, 5-Mazaculos II, 6-Riera, 7-Cueto La Mina, 8-Bricia, 9-Penicial, 10-Lloseta, and 11-Les Pedroses. Cue beach and Llanes meteorological station were also plotted.