Quaternary Geochronology 41 (2017) 37–50

Contents lists available at ScienceDirect

Quaternary Geochronology

journal homepage: www.elsevier.com/locate/quageo

Research paper

Inter- and intra-crystalline protein diagenesis in *Glycymeris* shells: Implications for amino acid geochronology

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ARTICLE INFO

Article history:
Received 26 September 2016
Received in revised form
23 May 2017
Accepted 23 May 2017
Available online 26 May 2017

Keywords:
Glycymeris
Mediterranean sea
Arrhenius parameters
Amino acid racemisation/epimerisation

ABSTRACT

This study focuses on the mollusc *Glycymeris*, which is commonly used to date raised marine deposits through amino acid racemisation (AAR). We examined the effect of bleaching and heating on the amino acid composition and racemisation/epimerisation of *Glycymeris* shells, as well as fossil material sourced from Spain. We show that amino acid composition differs between the umbo and rim zone of *Glycymeris*. This difference may be attributable to sampling strategies that target distinct microstructural zones. Also, the inter- and intra-crystalline proteins in the umbo appeared to be similar, as reflected by similar amino acid composition, which varied in a similar way with time when exposed to heating. We confirm that the intra-crystalline fraction in *Glycymeris* does not behave like a closed system, as the leaching of amino acids and D/L values showed similar variations in unbleached and bleached samples. We propose that the sediment matrix on which the molluscs were deposited influence amino acid racemization. In contrast, the matrix does not appear to have affected protein leaching/degradation. We measured Arrhenius parameters for Asx, Glx, and Ile in *Glycymeris*, finding them to be similar to those of other molluscs.

1. Introduction

The dating of raised Pleistocene marine deposits is of considerable interest in the Mediterranean basin. These deposits attest to the highstand sea level episodes that roughly coincided with interglacials and interstadials. Traditionally, these deposits have been dated through the palaeontological content, e.g. the presence of *Persististrombus latus* appeared to attest to deposition within the Eemian Stage (MIS 5e) (Dumas, 1981; Baena et al., 1981, 1982; Goy and Zazo, 1982, 1986, 1988; Somoza et al., 1987). However, it has been observed that this species also entered the Mediterranean Sea during Marine Isotopic Stage (MIS) 7 (Goy et al., 1986; Hearty et al., 1986; Hillaire-Marcel et al., 1986; Causse et al., 1993; Zazo, 1999; Zazo et al., 2003; Torres et al., 2006, 2010). U-series dating of mollusc shells has also been used to estimate age (Bernat et al., 1978; Dumas, 1981; Radke et al., 1981; Causse et al., 1993; Brückner, 1986; Goy et al., 1986; Hillaire-Marcel et al., 1986), but this method is considered unreliable because such shells are geochemically open systems (Kauffman et al., 1971; McLaren and Rowe, 1996).

Several studies have demonstrated that amino acid racemisation (AAR) is a satisfactory tool for dating palaeontological sites and raised marine deposits (Wehmiller, 1982; Miller and Mangerud, 1985; Goodfriend, 1992; Goodfriend et al., 2000; Murray-Wallace, 2000; Wehmiller and Miller, 2000), especially when it is combined with the use of the bivalve *Glycymeris* (Hearty, 1986, 1987; Hearty et al., 1986; Torres et al., 2000, 2010, 2013; Ortiz et al., 2004). AAR, which is a faster and less expensive technique than radiometric dating approaches, allows the age discrimination of fossil beach deposits, thus facilitating the identification of reworking and time-averaging (Martin et al., 1996; Kowalewski et al., 1998; Krause et al., 2010). Furthermore, 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.

Here we focused on sampling shells belonging to the pelecypod *Glycymeris* sp. This mollusc was chosen because it is widely represented in the raised marine deposits of the Mediterranean realm and it has been shown to provide useful AAR data. The use of aminozones, using D-alle/L-Ile values measured in *Glycymeris* shells, has enabled a general application for the Mediterranean (Hearty et al., 1986; Hearty, 1987; Torres et al., 2000). In some cases,

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http://dx.doi.org/10.1016/j.quageo.2017.05.007
1871-1014/

age calculation algorithms for aspartic acid, glutamic acid, leucine, phenylalanine, and isoleucine have also been defined (Ortiz et al., 2004).

Torres et al. (2013) concluded that isoleucine epimerisation ratios in *Glycymeris* have a greater capacity to discriminate ages of sites than glutamic acid and aspartic acid. This study also showed that time-linked taphonomical processes and time-averaging produce high variability in D/L values, thus hindering the identification of substages in stacked shell beds in raised beach deposits belonging to the same MIS.

Recently, Demarchi et al. (2015) examined the intra-crystalline fraction of proteins in the rim of *Glycymeris* shells, concluding that this fraction does not behave as a closed system. They also observed a similar behaviour between D/L values in bleached and unbleached samples. However, they concluded that the rim of the shell should be used with caution for dating by AAR—a conclusion that was consistent with the findings on unbleached *Glycymeris* (e.g. Hearty et al., 1986), and according to Torres et al. (2013), *Glycymeris* show intrashell variability. Therefore, it is necessary to sample the same part of the shell, namely the complex cross lamellar region near the umbo.

However, some uncertainties regarding the protein diagenesis of *Glycymeris* shells remain. Further research is therefore required to clarify the processes of protein preservation and degradation, and the concomitant performance of AAR for dating sites using this species.

Here we examined the amino acid content and D/L values in the umbo of modern *Glycymeris* shells. For this purpose, we addressed 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. Additionally, we induced artificial diagenesis in proteins (both inter- and intra-crystalline amino acids, and the isolated intra-crystalline fraction) of shells by exposing them to high temperatures (80°, 110°, and 140 °C) over a range of times (0–5738 h). Protein breakdown was quantified by measuring the extent of racemisation of various amino acids. This approach provided data on protein diagenesis in modern *Glycymeris* shells. For comparison purposes, we also examined the whole protein content in fossil *Glycymeris* of various ages (MIS 1, 5, 7 and 11).

2. Material and methods

We measured the amino acid content of modern *Glycymeris nummaria* (Linneo 1758; sin. *G. insubrica* Bocci 1814, and *G. violascens* Lamarck 1819) shells obtained from a fish market. From the raised marine deposits, *G. nummaria* shells also accounted for most of the shells sampled (see Supplementary Information). Nevertheless, we refer to all species as *Glycymeris* sp., as do other authors (cf. Belluomini et al., 1986, 1993; Hearty, 1986, 1987; Hearty et al., 1986; Torres et al., 2000, 2010, 2013; Ortiz et al., 2004).

In the laboratory, shells were carefully sonicated and cleaned with water to remove sediment. Peripheral parts, approximately 20–30%, were removed after chemical cleaning of the sample with 2 N HCl.

For all samples, we drilled a small disc in the umbo—a procedure reported to reduce variability within sample (Murray-Wallace, 1995). The selection of this site was also based on the results from the petrographic analysis, which showed that the inner part of the shell (complex crossed lamella), near the umbo, is made of calcite (cf. Torres et al., 2013; Demarchi et al., 2015).

We subjected *ca.* 5–20 mg of sample to AAR analysis in order to determine total protein content (inter- and intra-crystalline proteins). Samples were also used to measure the amino acids in the intra-crystalline fraction after bleaching. Additionally, samples were subjected to leaching experiments at three temperatures. They were then analysed for their total amino acid content and the intra-crystalline protein fraction after bleaching.

We also considered the AAR values of *Glycymeris* shells from raised beach deposits of diverse ages off the Mediterranean coast of Spain (Fig. 1), previously analysed by Torres et al. (2010, 2013): GA-18, FAR-C, RM-4, LMR, GA-2 (Table 1). For these sites, we considered only analysed by liquid chromatography, discarding gas chromatography results as some amino acids, such as serine, are not identified using this technique.

2.1. Leaching experiments

To mimic all the taphonomical conditions of the outcrop, heating experiments were performed using two sediment matrices, namely calcite and quartz sand. Carbonate matrices are predominant in almost all outcrops, as attested by carbonate bioclasts, rock fragments and cement. Quartz matrices are predominant in uncemented ancient beach deposits, in which rock fragments are of siliceous nature.

A set of samples and 2 g of quartz sand (deeply pre-cleaned by oven baking at 600 °C for 6 h) were placed into borosilicate glass ampoules. Another set of samples with 2 g of pure powdered calcite sand was also placed into borosilicate glass ampoules. Calcite sand was obtained after crushing a big calcite crystal (Iceland spar variety) from a hydrothermal vein.

Next, 120 mL of ultraclean water (grade HPLC) was added via a syringe. The top of the ampoule was fitted into rubber tubing connected to a vacuum-N_2 line, being alternately exposed to vacuum and to N_2, repeated three to four times to flush out all the air. The ampoule was later sealed under nitrogen. The ampoules were placed in a rack and put into an oven at various temperatures (140 °C, 110 °C and 80 °C) for different times (Table 2).

Thus, two ampoules with quartz sand and another two ampoules with calcite from each temperature experiment were removed at the intervals shown in Table 2. The ampoules were opened and dried. Shell samples were separated, washed with distilled water, sonicated, and vacuum-dried.

Samples were then analysed for total amino acid content and intra-crystalline fraction after bleaching.

2.2. Bleaching

Powdered samples from the umbo of the same *Glycymeris* shells used to analyse the total protein content were also used to isolate intra-crystalline proteins. Following Demarchi et al. (2013a), the shell particles measured less than 500 μm, a size for which bleaching is most effective. In this regard, we exposed these powdered samples to 10% sodium hypochlorite (NaOCl), an effective oxidising agent for this purpose (Penkman et al., 2008; Demarchi et al., 2013a). Samples were exposed to NaOCl for 48 h, a time reported to be the optimal bleaching period for molluscs (Penkman et al., 2008; Demarchi et al., 2013a, 2015).

For each fraction, 50 μl of NaOCl per mg of powdered shell was added. To ensure the complete penetration of the oxidising agent, the vials containing the powders and the bleach were shaken every 24 h. The bleach was then removed, and the powders were rinsed five times in ultrapure water and once in HPLC-grade methanol, with centrifugation for 4 min between each rinse to minimise the removal of powder. Finally, the samples were air-dried overnight.

2.3. Amino acid analysis

Amino acid concentrations and racemisation/epimerisation ratios were quantified using HPLC, following the sample preparation