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Proteinaceous Material from Potsherds and Associated Soils

R. P. Evershed

University of Bristol, School of Chemistry, Cantock's Close, Bristol BS8 1TS, U.K.

Noreen Tuross

Conservation Analytical Laboratory, Smithsonian Institute, Washington DC, U.S.A.

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Amino acid and protein analyses of a range of archaeological potsherds and associated burial soils were performed by direct hydrolysis of powdered soils and potsherds samples, followed by high performance ion exchange chromatography. Detection of the eluting amino acids was by post-column derivatization, using o-phthalaldehyde and fluorescence monitoring. Microgram amounts of hydrolysed amino acids were detected in all the potsherds and soils analysed; free amino acids in both potsherd and soils were often below detectable limits (sherds recovered from the same archaeological context). The protein analyses involved extraction of powdered soil and potsherd samples with urea buffer, followed by polyacrylamide gel electrophoresis and protein staining. High sensitivity detection of the proteins used gold staining, following electrotransfer on to nitrocellulose. No obvious protein bands of molecular weight >10 kDa were detected in any of the potsherd samples that were studied. In general the amino acid and protein compositions of the individual potsherds were not sufficiently characteristic to suggest that HPLC "fingerprinting" of amino acids would significantly contribute to palaeodietary analysis or vessel use. Two potsherds, however, exhibited evidence of gelatin/collagen amino acid patterns. The presence of small amounts of gelatin were confirmed by the digestion of a >10 kDa protein extracted by bacterial collagenase. © 1996 Academic Press Limited

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Introduction

It is now well established that lipids survive in association with ancient potsherds (for review, see Heron & Evershed, 1993). Lipids have been detected in archaeological ceramics by means of a number of chromatographic and spectroscopic techniques, including: thin layer chromatography, infrared spectroscopy, gas chromatography (GC; Condamin *et al.*, 1976; Patrick, Koning & Smith, 1985; Evershed, Heron & Goad, 1990; Skibo, 1992); combined gas chromatography/mass spectrometry (GC/MS; Evershed *et al.*, 1990); pyrolysis-GC/MS (Oudemans & Boon, 1991); and isotope ratio monitoring GC/MS (Evershed *et al.*, 1994). Both visible surface and absorbed residues from archaeologically recovered pottery have been examined for lipid content. Recent work (Heron, Evershed & Goad, 1991; Evershed *et al.*, 1994) has established that the transfer of lipids between potsherds and their associated burial soil is minimal in freshly excavated potsherds and burial soils freed from the potsherd surface. Separation of derivatized lipid by GC and identification of the extracted material by GC/MS were used to compare the lipid content of the soils and potsherds both quantitatively and qualitatively. The results indicate that in the majority of cases, clear distinctions can be drawn between the lipid content of the soil, arising from the decay of plants, animals and micro-organisms, and the lipids present in the potsherds, presumed to have been absorbed during the use of the vessels.

Moreover, there is evidence that the lipid components of the organic residues of the potsherds can be related to waxes and fats deriving from the plant and animal matter that had come into direct contact with the archaeological vessels during their use (Evershed *et al.*, 1991, 1992, 1994). The appreciable quantities of lipids absorbed in vessels (Charters *et al.*, 1993) provides evidence for a transfer mechanism and deposition of lipids in the ceramic fabric. The favourable survival of lipids in association with archaeological ceramics can be attributed to their inherent hydrophobicity, which will limit their movement from their original site of deposition due to the action of percolating ground waters (Evershed, 1993).

Although data have accumulated on the survival of lipids in pottery, there are surprisingly few reports on the occurrence of other classes of compounds, e.g. proteins and carbohydrates, the major molecular constituents of plants and animals. There is every

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reason to believe that proteins and carbohydrates will also be released from foodstuffs during cooking and then possibly absorbed by the porous ceramic fabric of unglazed pottery vessels. However, it might be expected that these more polar compounds would be more readily lost from potsherds, or perhaps more likely, would equilibrate with the surrounding environment (soil) during burial. Significantly, proteins and carbohydrates are generally considered to be intrinsically less resistant to degradation compared to lipids (Tegelaar, 1990; Whelan & Emeis, 1992).

Evidence that proteins survive in archaeological materials comes from the facile recovery of collagen and its routine use for radiocarbon dating (e.g. Vogel & Waterbolk, 1963; Longin, 1971; Stafford *et al.*, 1987) and the determination of δ^{13}C and δ^{14}N values in palaeodietary investigations (e.g. Koch, Fogel & Tuross, 1994). The survival of collagen in calcified tissue is enhanced by this protein's insolubility in aqueous solution. Although less is known about non-collagenous proteins in bones and teeth, the mounting evidence indicates that certain proteins can survive burial for extended periods of time (Smith & Wilson, 1990; Cattaneo *et al.*, 1990; Tuross, 1991; Aije *et al.*, 1991; Tuross & Stathmoplos, 1993). Incubation of synthetic hydroxyapatite with human blood demonstrates tight association of several plasma proteins with the calcium phosphate matrix (Tuross, 1993). Survival of protein in an archaeological setting, exclusive of calcified tissue, has been most intensively studied on stone tool residues. Several reports indicate sporadic preservation of very small amounts of protein on lithic edges (Lowenstein & Scheuenstuhl, 1991; Cattaneo *et al.*, 1993; Tuross & Dillehay, in press). These findings contrast studies utilizing haemoglobin crystallization and crossover immunoelectrophoresis, that suggest high (μg) amounts of proteins survive on stone tools (Newman & Julig, 1989; Loy & Wood, 1989).

In this study, results of protein and amino acid analyses of an assemblage of Late Saxon/early mediaeval potsherds and associated burial soils are presented. These samples have already been subjected to lipid analyses (Heron *et al.*, 1991; Evershed *et al.*, 1994), and the benefit of additional information has been considered. Replica pottery vessels used in experimental simulations were also used in this study.

On the basis of these results, it is concluded that protein and amino acid analyses are unlikely to yield simple, straightforward data, either by means of protein identification methods or building block amino acid chemistry. However, trace amounts of the abundant animal protein collagen, in the form of gelatin, are present in some of the pottery examined. This study continues to support the view that differential preservation of lipids versus protein does exist in adsorbed pottery residues. The enhanced preservation of lipid is presumed to reflect the greater susceptibility of protein and amino acids to the effects of heat of cooking, the relatively limited transfer to clay matrices, and the leaching of hydrophillic molecules.

Materials and Methods

Sample

All the archaeological samples (soils and potsherds) were of Late Saxon/early mediaeval date, collected from the West Cotton site, Raunds, Northamptonshire, U.K. As full details, i.e. excavation context, grid references and descriptions (ceramic and context) were given in our earlier publication on this subject (Heron *et al.*, 1991), these will not be repeated here. The potsherds studied were WC06, WC09, WC21, WC38, WC48, WC66, WC69; the soils included WC06, WC09, WC48, WC57, WC66, 170/593, 187.3/645 and 187/643.5. For comparative purposes, analyses were performed on body potsherds taken from two experimental vessels that had each been used on separate occasions to boil cabbage and lamb. Further details of these cooking experiments, using replica vessels, will be presented elsewhere (Evershed *et al.*, in press; Charters *et al.*, in press).

Sample preparation

The soil was removed from the surface of the potsherds with a sterile spatula and ground to a fine powder, using a pestle and mortar. The surface of each potsherd, including the fractured edges, was cleaned, using a modelling drill, to remove all traces of adhering soil. A fragment of the potsherd was removed and crushed to a powder, using a pestle and mortar.

Protein hydrolysis/amino acid extraction

Powdered soils or potsherds (8–12 mg) were weighed into hydrolysis tubes (13 × 100 mm; Kimax, IL, U.S.A.), 200 μl of 6 N hydrochloric acid added, the tubes purged with nitrogen, sealed and heated (100°C, 20 h). After heating, the tubes were cooled, opened, and the hydrochloric acid removed under a stream of blown nitrogen (50°C). Once dried, 200 μl of water were added and the solutions again blown to dryness. Prior to HPLC, the dried residues were dissolved in water (to give concentrations equivalent to 10 mg ml^{-1} of the original potsherd or soil) and filtered through 0·45 μm filters (Millipore, Yonezawa, Japan) to remove suspended particulate matter.

Extraction of free amino acids

Powdered soils or potsherds were weighed (c. 10 mg, accurately weighed) into sterile glass tubes and extracted (overnight, room temperature) with pH 2 buffer or 3 N HCl at a soil/potsherd weight:volume ratio of 10 mg ml^{-1}. The supernatants containing the free amino acids were filtered, as described above, prior to

ion exchange HPLC. Experimental vessels that had not been used in cooking experiments were incubated in a solution of free amino acids (Sigma Chemical Co., U.S.A.) and both pH 2 buffer and 3 N HCl were able to recover the doped amino acids.

Ion exchange chromatography

Amino acid analyses were performed according to the method of Hare, St John and Engel (1985). The filtrate (20 μl) from above was introduced via a Rheodyne loop injector into a 10 cm × 0·2 cm i.d. column packed with 2·4 μm cation exchange resin (St John Associates Inc., U.S.A.). Detection of the eluting amino acids was achieved by fluorescence monitoring of a post-column addition of a mixture of o-phthalaldehyde and 2-mercaptoethanol in a potassium borate buffer. Very sensitive detection of the amino acid derivatives was achieved, using the excitation wavelength of 369 nm with monitoring of the fluorescence above at 455 nm (Darbre, 1986). Although this method of amino acid analysis cannot recognize secondary amines such as proline and hydroxyproline, its major advantage is the ability to inject directly extracts of archaeological materials such as pottery and soils and thus achieve quantitative recovery of primary amines. Peak assignments were made by retention time comparison with a mixture of authentic amino acids (Sigma Chemical Co., U.S.A.) analysed under the same chromatographic conditions.

Protein extractions

Samples (1 g) of finely ground soils and potsherds were extracted (shaken overnight, room temperature) with 7 M urea (1·5 ml). After extraction, the solutions were centrifuged (15,000 r.p.m.) to remove suspended particulate matter. The supernatants were then concentrated to c. 100 μl, using Centricon (Amicon, MA, U.S.A.) concentration tubes (10 kDa cut-off filters). Aliquots (25 μl) of these extracts were electrophoresed (Laemmli, 1970) on SDS polyacrylamide gels (4–20% gradient, Novex, CA). After electrophoresis, proteins were electrotransferred (Towbin, Stachlin & Gordon, 1979) to nitrocellulose. The polyacrylamide gels and nitrocellulose membranes were visualized by staining with Coomassie blue and colloidal gold, respectively. Two archaeological potsherds (WC09 and WC38) were extracted in 5 g portions in 7 M urea. The urea extractant was then reduced in volume over centricon Ym 10 (10 kDa cut-off), exchanged into water and one third of the retentate was analysed for its hydrolysable amino acid content. The remaining sample was divided in half and diluted with X2 collagenase buffer (Tuross & Stathoplos, 1993). One sample was treated with bacterial collagenase (Advanced Biofractures) and the other used as a control. Both samples were then put over Ym 10 centricons and then the pass through liquid subject to amino acid analysis.

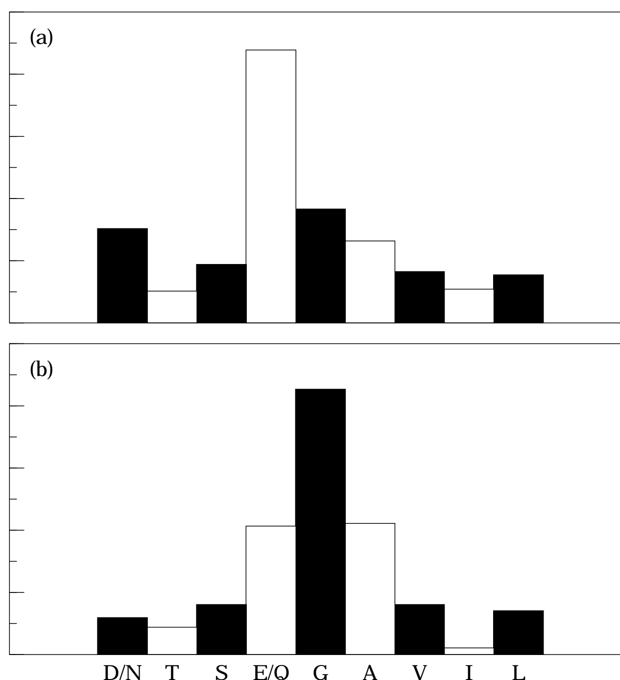

Figure 1. Distribution of amino acids in experimental pottery that had been used 10 times in cooking cabbage (a) and lamb (b). The amounts are scaled to the most abundant amino acid in each sample and do not reflect quantitative recoveries. The differences in the distribution of the amino acids is pronounced with glycine the predominant amino acid in the lamb cooking pot, and glutamic acid the most abundant in the cabbage cooking pot. We note the large amounts of aromatic amino acids in these pots is an unexpected result. D: aspartic acid; N: asparagine; T: threonine; S: serine; E: glutamic acid; Q: glutamine; A: alanine; V: valine; I: isoleucine; L: leucine.

Results

Various approaches were adopted to investigate the presence of proteins and amino acids in archaeological ceramics. The primary aim was to establish whether proteins or amino acids survive in archaeological ceramics and if they can be distinguished from the proteinaceous material present in the burial matrix (soil). Parallel analyses were performed on archaeological soils samples recovered either directly from the surface of the pottery, or from soil taken from the same archaeological context of recovery as the potsherds.

Amino acid analysis

Experimental pottery. The analytical baseline for this study was the determination of free and peptide bound amino acids in two experimental clay vessels: one that had been used 10 times in cooking cabbage, and the other that had been used five times with lamb. The unused pottery served as the control. Free amino acids from all of the experimental vessels and hydrolysed amino acids from the unused vessels were either below detectable limits of the analyses or at very low (femptomole) levels. In contrast (Figure 1), the amino

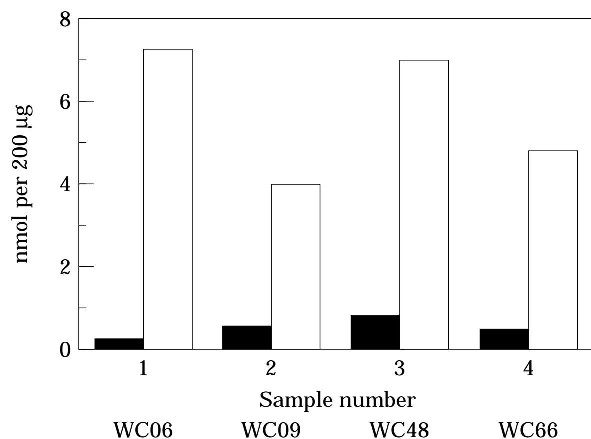

Figure 2. The hydrolysable amino acid content in paired soil/potsherd samples from 200 μg dry weight of sample. The soil samples ranged from three to eight times the amount of hydrolysable amino acids compared to those found in associated potsherds. The amounts reported are based on the size of samples that were feasible to inject into the ion exchange HPLC. The data suggest non-equilibrium conditions between the soils and potsherds. ■: sherd; □: soil.

acid analyses from the two cooking vessels revealed different distributions of adsorbed amino acids. Glycine, the predominant amino acid found in collagen, was also the dominant amino acid in the lamb vessel hydrolysate. The cabbage cooking vessel exhibited a different distribution of amino acids, with glutamic acid more abundant than the other amino acids (Figure 1). It should be stressed that these peptide bound amino acids must have been adsorbed from the food used in cooking, as the experimental vessels were not handled after firing, and no burial was involved.

Archaeological pottery. In the amino acid profiles of paired potsherd/soil samples, three results emerged: (1) quantitatively, on a dry weight basis, there were consistently more hydrolysable amino acids in the soil as compared to the potsherd (Figures 2 & 3);
(2) soil patterns were qualitatively different from potsherd contents (Figures 3 & 4), with a tendency towards enrichment of the acidic amino acids, principally aspartic acid, in the soil hydrolysates;
(3) free amino acid contents in both soils and pottery were, as in the experimental pots, extremely low (femptomole to low picomole) under extraction conditions that did recover free amino acids from ''spiked'' pottery (see Materials and Methods).

The yield of hydrolysable amino acids shown in Figure 2 ranged from approximately 4–8 nmol per 200 μg of dry soil, compared to one or less nmol per equivalent weight of pottery. This pattern suggests that the peptides and protein in soil and pottery have not come to equilibrium. In addition (Figure 3), representative chromatograms of the acidic and neutral amino acids (aspartic acid to valine) illustrate the

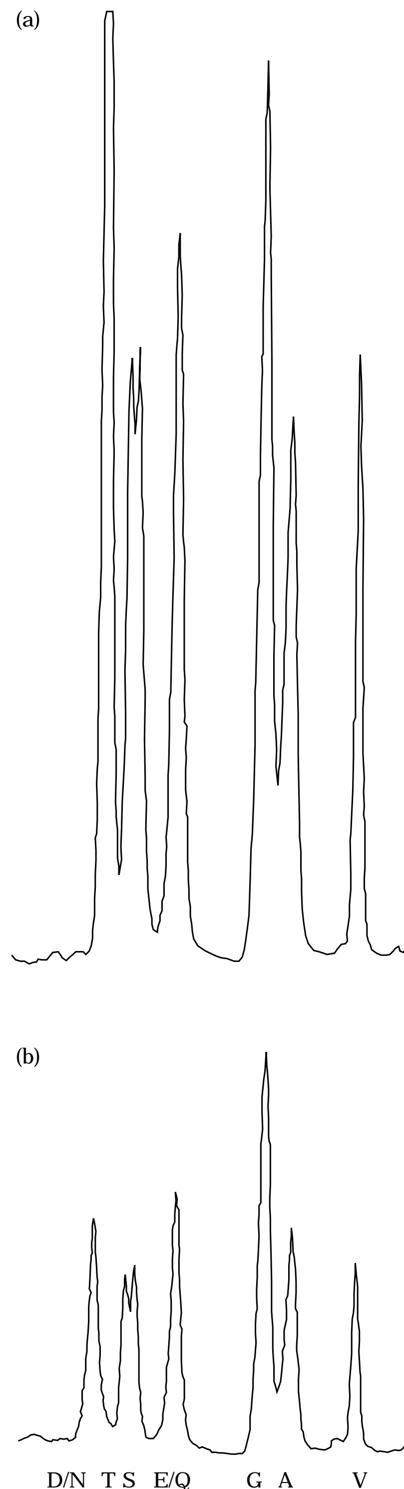

Figure 3. Partial amino acid chromatograms of (a) soil and (b) potsherd. The acidic and neutral amino acids, ranging from aspartic acid to valine, are shown. An equivalent hydrolysate of 200 μg of sample was injected, separated by cation exchange chromatography and reacted with the fluorescent reagent o-phthalaldehyde (see Materials and Methods). The chromatograms are shown at the same attenuation, and the difficulty in resolving theonine and serine derives from the high salt content of the samples. Key same as for Figure 1.

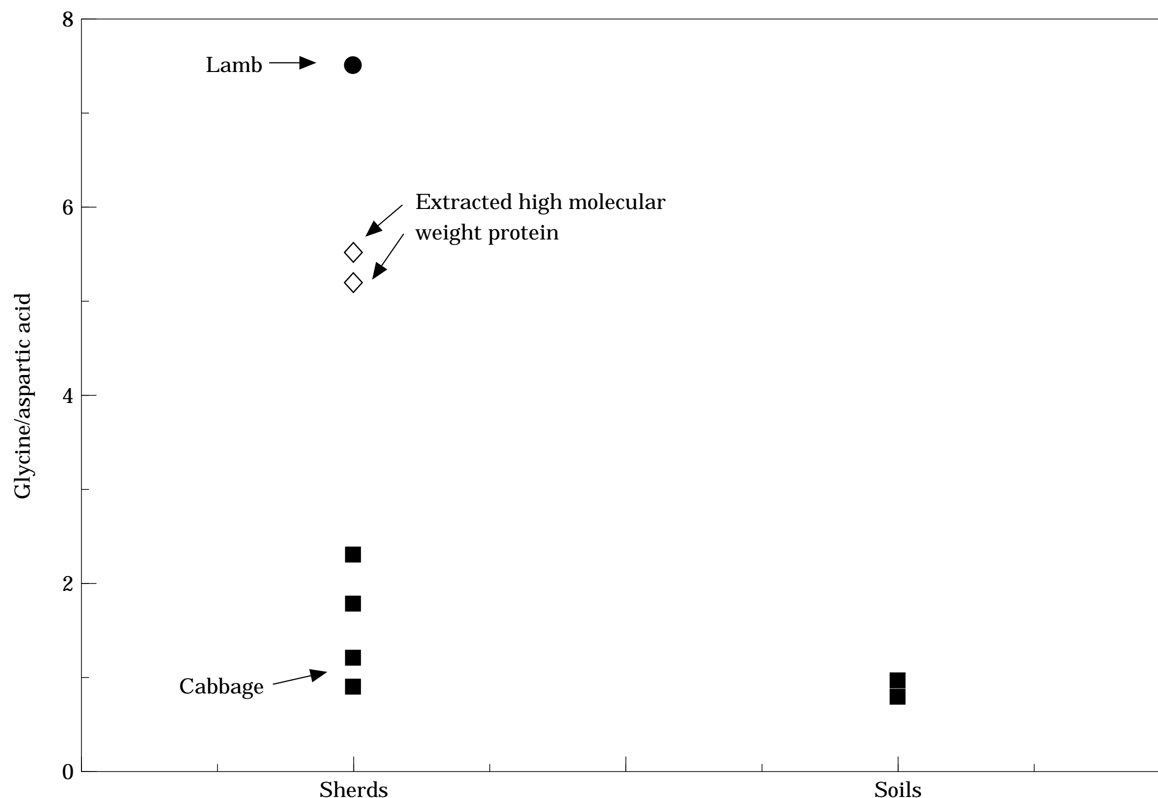

Figure 4. Glycine to aspartic acid ratios of total hydrolysates of experimental vessels (annotated cabbage and lamb), archaeological pottery (solid squares) and high molecular weight extracts from two of the archaeological pots, WC09 and WC38 (open diamonds). Collagen gly/asp is in the range of 6 to 7 (Tuross *et al.*, 1988).

qualitative differences found in the soil versus pottery. We have chosen to base our analyses on these first seven amino acids because they comprise over 85% of the total amino acid content [as determined by post-column OPA (see Materials and Methods)], and because of interferences, such as glucosamine, in the soil and high concentrations of ammonia, which make the later portions of the chromatograms difficult to interpret reliably.

The tendency towards elevated glycine levels in the pottery and increased content of the acidic amino acid, aspartic acid, in the soil is seen in Figure 4. Eight soil samples cluster tightly in glycine to aspartic acid ratios between 0·75 and 1, while the potsherds ranged from approximately 0·9 to 1·8. It is tempting, based on the experimental pottery data and the higher glycine content observed in the lamb cooking vessel, to suggest that elevated glycine content in some of the archaeological pottery may derive from collagen, but differential binding of clay versus soil particles is another reasonable hypothesis.

Protein extraction and analysis

Protein recovery tests. In order to determine whether, experimentally, intact protein could be recovered from the pottery clay matrix, bovine serum albumin was chosen as a model protein and added to powdered ceramic known from amino acid analyses (see above) to be free of protein. After air drying the protein-"spiked" ceramic, the clay was extracted in 7 M urea. This denaturant was used because it is capable of solubilizing resistant protein and can be directly electrophoresed in sodium dodecyl sulphate (SDS) acrylamide gels. As shown in Figure 5, a clear doublet can be seen, corresponding in electrophoretic mobility to BSA in several dilutions. On this basis, we would predict that low ng levels of intact protein could be recovered and visualized by these methods.

Archaeological pottery. Ten pottery samples (approximately 1 g) were extracted in 7 M urea; the supernatant concentrated to a small volume, and electrophoresed under the same conditions as seen in Figure 5. No detectable bands were seen, either with direct staining of the gel with Coomassie Brilliant Blue, or after transfer of the extracted material to nitrocellulose and subsequent staining with colloidal gold. Thus, the peptides/protein that were observed by amino acid analysis were presumed to be in the form of breakdown products and no "intact" protein product was present at greater than low ng quantities.

Two samples of potsherds (WC09 and WC38) were available in larger quantities and approximately 5 g of

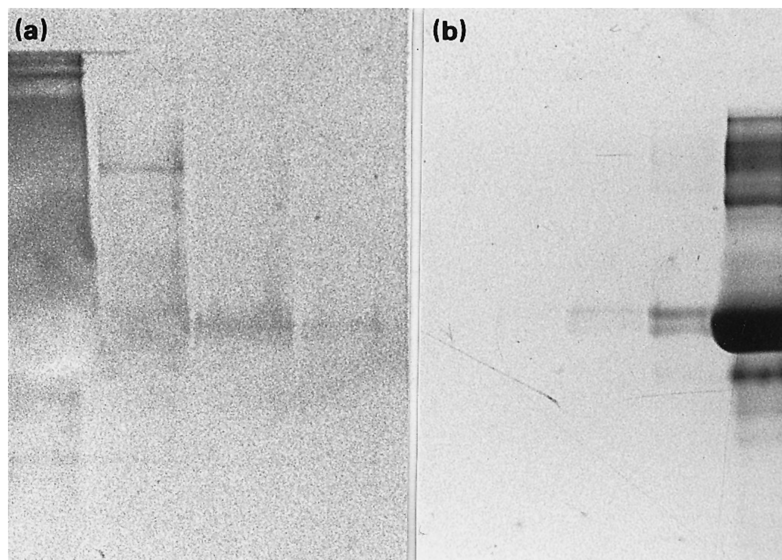

Figure 5. Mirror image photographic records of the same extraction of albumin from the ground clay matrix. The protein was added to sterile clay and removed with 7 M urea. The extracted material was separated by electrophoresis on acrylamide and then transferred to nitrocellulose by electrolution. (a) Gold stained transfer; (b) Coomassie blue stained gel. Centre lanes [extreme left of (a) and extreme left of (b)] from each support illustrate the limits of each technique: 10 ng of material are fully visible in the gold stained blot, but are below detection limits of Coomassie staining directly only the gel.

each pot were extracted in 7 M urea and the supernatant reduced to a small volume over a filter that retained 10 kDa material. After exchanging the urea into water, the amino acid analysis of one third of the extract of WC09 is shown in Figure 6(a) compared to the total hydrolysable amino acid pattern of this pot; the extracted high molecular weight material is even more enriched in glycine, i.e. the glycine/aspartic acid ratio increased (cf. Figure 4). In addition, and most conclusively, this extracted, high molecular weight material was shown to be digested by bacterial collagenase by assaying the amino acids that passed through a 10 kDa filter after exposure to this enzyme. In the absence of bacterial collagenase, virtually no protein was recovered in the pass through (see Materials and Methods), while the collagenase samples had amino acid analyses similar to those shown in Figure 6. The close similarity between the amino acid profiles for gelatin extracted from the archaeological sample WC09 and the total hydrolysable amino acid extract of the lamb pot is shown in Figure 6.

We would stress that the amount of gelatin found in the association with this archaeological pottery is small (in the range of 50–100 pg g^{-1} pot). Given that gelatin, by definition, consists of a range of molecular weight degradation products generated from collagen, direct visualization by electrophoretic methods is not generally feasible.

Discussion and Conclusions

We present here the results of the first systematic study of proteinaceous materials associated with archaeological ceramics. Experimental pottery vessels used in meat and vegetable cooking supply evidence that proteinaceous material can be transferred to clay, and that a characteristic amino acid signature can result when foodstuffs are processed. Deconvoluting back from the amino acid patterns of adsorbed residues in archaeological pottery to ancient food utilizations will be possible only in selected cases, due to heterogeneity, transfer and taphonomic issues. Although this study does not address the unique aspects of clay chemistry that are likely to impact protein preservation in the archaeological record, we should be aware that the pH of water at the clay mineral surface can be substantially more acidic (4–5·5 pH units) than the bulk water (McCabe, 1992). These strongly acidic conditions would not bode well for the long-term preservation of proteinaceous materials.

The data reported here, both from experimental vessels and from archaeological potsherds, indicate that very little protein or peptide material is likely to be incorporated into the clay matrix of pottery. Although these data suggest that protein analyses may contribute to the overall picture of pottery usage, especially as regards the cooking of animal products, the concentrations of peptides and amino acids found in this archaeological pottery are very much less (*c.* 10,000 times) than lipids associated with the same samples (Charters *et al.*, 1993). Gelatin produced in large quantities in the cooking of most animal products, may prove to be rather easier to identify than other remnant proteins, largely because of its characteristic amino acid composition, its transition from soluble to colloidal form upon cooling, and its susceptibility to

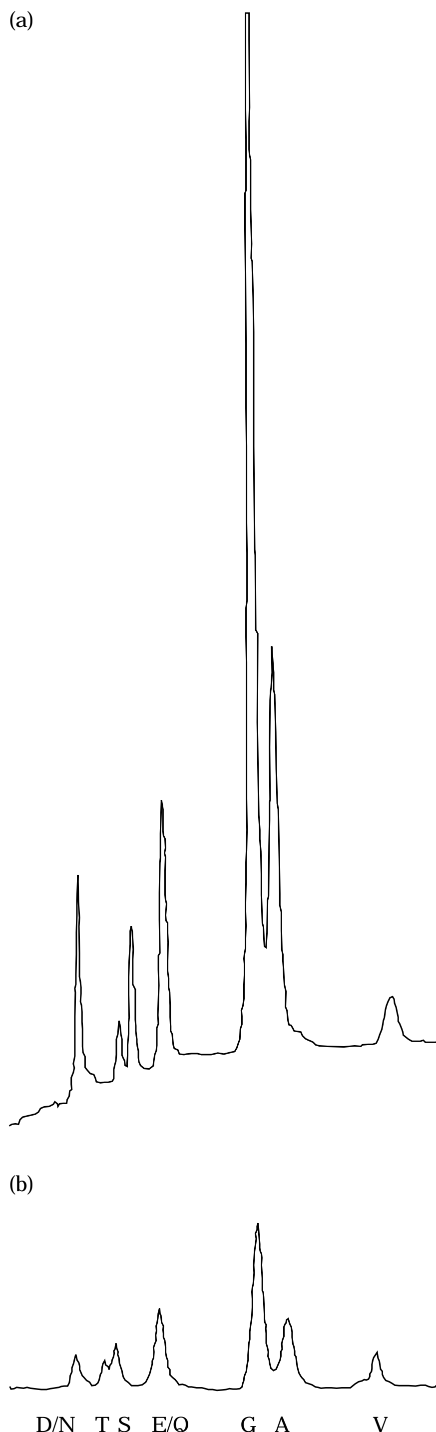

Figure 6. Partial amino acid chromatogram of (a) extracted high molecular weight (10 kDa) protein found in association with potsherd WC09, and (b) the total hydrolysate of the experimental pot that had been used in cooking lamb. Key same as for Figure 1.

experimental cleavage by a unique set of enzymes. The demonstration of small amounts of gelatin, and its association with animal products in archaeological pottery are, nonetheless, important additions to the complex investigations of pottery utilization. It is noteworthy that the gelatin was recovered from two potsherds that yielded high concentrations of saturated fatty acyl lipids. Both these findings point to a previous association of the pot from which the potsherds were derived with the processing of meat products. Recent developments in the sensitivity of on-line gas chromatography/isotope ratio mass spectrometry remove the requirement for carbonized food encrustations (Skibo, 1992) in order to produce light isotropic data from pottery residues (Freeman *et al.*, 1990; Silfer *et al.*, 1991; Johnson, Fogel & Miller, 1993; Merritt & Hayes, 1994; Evershed *et al.*, 1994). In contexts where foodstuffs contribute isotopic signatures that can be distinguished from soil background, or where amino acid-lipid spacings prove informative, the future of the analyses of proteins in pot residues may reside in their isotopic values.

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