[RADIOCARBON, VOL. 34, NO. 3, 1992, P. 292–295]

EXPERIMENTS ON ^{14}C DATING OF CONTAMINATED BONE USING PEPTIDES RESULTING FROM ENZYMATIC CLEAVAGE OF COLLAGEN

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ABSTRACT. We describe the use of collagenase to digest ancient collagen samples to obtain a peptide mixture, and the use of reversed-phase chromatography to purify these peptides. The isolation of the tripeptides glycine-proline-hydroxyproline and glycine-proline-alanine is especially encouraging.

INTRODUCTION

The use of enzymes, quite common in biochemical procedures, is not yet established in ^{14}C chemistry. Recent carbon-containing proteins, such as enzymes, show modern ^{14}C activities, and thus, can quite easily contaminate samples. However, the advantages of their use are considerable; enzymes require a specific substrate, and reactions are mostly quite specific, resulting in chemically well-defined reaction products. The latter would be especially advantageous for dating contaminated bone (Hedges & van Klinken 1992). So far, enzymes have been used with encouraging results in stable isotope chemistry (DeNiro & Weiner 1988; Schimmelmann & DeNiro 1983).

In this paper, we briefly describe the isolation of small peptides from collagen after digestion with clostridial collagenase, and purification of the peptides using reversed-phase chromatography. We used previously dated bone samples that had yielded dates contrary to archaeological expectation, and thus, might have been contaminated with exogenous material. First, we isolated specific molecular-weight fractions of both the cold, weak acid-insoluble fraction (demineralization pellet) and soluble fraction (demineralization supernatant) using ultrafiltration. Ultrafiltration devices are far more easy to handle, and quicker, than dialysis tubes (Brown *et al.* 1988). This step enabled us to compare, for example, high- and low-molecular-weight fractions in C/N ratio, $\delta^{13}C$ and ^{14}C age. We then used the >10 kD (>10,000 Dalton = amu) fractions in the enzyme digestions. During enzymatic treatment, the collagen α-chain was cleaved mainly into tripeptides, *e.g.*, glycine-proline-hydroxyproline (Gly-Pro-Hyp; 10% of the collagen α-chain consists of this sequence), and glycine-proline-alanine (Gly-Pro-Ala; 7%), and many others. After enzymatic cleavage, we separated the peptides according to hydrophobicity using reversed-phase chromatography. If we can show that this type of separation (which employs organic buffers) does not interfere with ^{14}C dating, another mode of separation can be added to the ion-exchange techniques currently used in ^{14}C chemistry. The employed buffers are volatile, and thus, facilitate easy recovery of peptide fractions by freeze-drying, without the need for desalting steps.

MATERIALS AND METHODS

Figure 1 shows how we obtain bone organic fractions; where possible, we use both the acid-insoluble and soluble fractions. They undergo basically the same treatment, except that the insoluble fraction is first gelatinized (gelatin 1), a step unnecessary for the acid-soluble solution (gelatin 2). Centrifuged solutions of gelatin 1 and 2 are then ultrafiltered, using Amicon Centriprep (up to 15-ml solutions) or Millipore Ultrafree-CL (up to 2-ml solutions) ultrafilters with molecular weight cutoffs of 10 kD or 30 kD. The retentates (the >10 kD or >30 kD fractions) are then digested using 1000 units (1 unit liberates peptides from collagen equivalent in ninhydrin color to 1.0 μmole of leucine in 5 h at pH 7.4 at 37°C in the presence of calcium ions) of clostridial col-

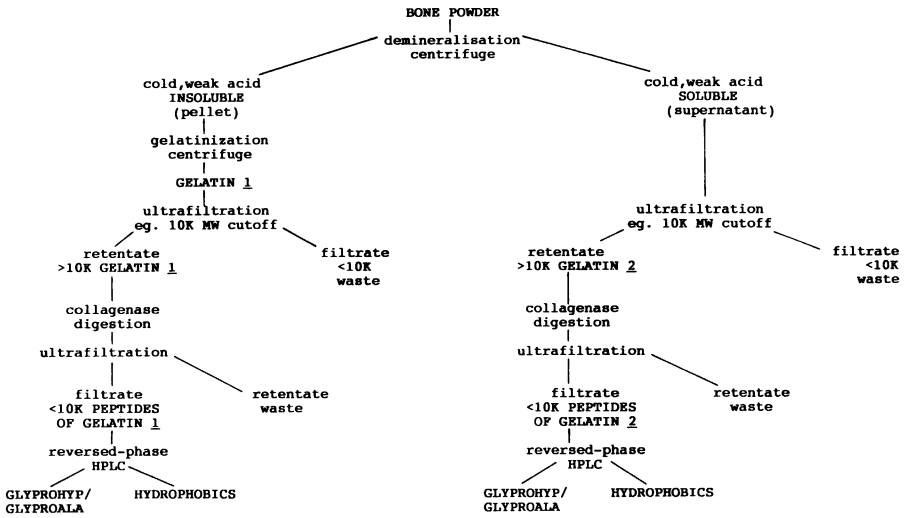

Fig. 1. Scheme of the chemical procedure

lagenase (Sigma Type VII high-purity collagenase) per 15 mg collagen in a 0.05 M Na phosphate, 0.36 mM $CaCl_2$, pH 7.5 buffer (DeNiro & Weiner 1988) at 37°C for 5 h. After enzymatic treatment, the solution is ultrafiltered again, this time, collecting the filtrate (*i.e.*, the <10 kD peptide fraction). Further details will be published in van Klinken and Hedges (ms.).

Part of the peptide mixture is then fractionated by reversed-phase HPLC, using a preparative Merck Lichrosorb RP-8 7-μm column (10 × 250 mm). Solvent A is a 6 mM HCl solution in water, solvent B is 6 mM HCl in acetonitrile (HPLC grade). The gradient is 0–5% B in the first 8 min, then 5–50% B in the next 20 min. Amino-acid analyses, comparison with the retention times of pure peptides (obtained from Sigma), and assessment of collected fractions by ion-exchange chromatography proved that the first peaks contain the tripeptides, Gly-Pro-Hyp and Gly-Pro-Ala. These were collected as one fraction (Fig. 2), as well as the more hydrophobic fraction that contains many different peptides (>50 peaks can be observed).

Results and Discussion

Table 1 gives the preliminary results of the measurements on the bones. Pestera PCR 3 (Groningen date, on gelatin (Longin extract): >46 ka BP, GrN-13,000), was put in to establish the background levels of the subsequent steps in the procedure. C/N ratios and $\delta^{13}C$ of the peptide mixture, and of the Gly-Pro-Hyp/Gly-Pro-Ala fraction after reversed-phase HPLC are both as expected; however, the hydrophobic fractions show much higher C/N ratios (3.4 and 4.8) and much more negative $\delta^{13}C$ values (−22.5 and −36.0‰). The (machine-)background-corrected ^{14}C dates of the different fractions show decreasing age with increasing hydrophobicity, the latter is implied by longer retention times during the reversed-phase separation. This effect is absent in the other bones we have analyzed. A possible explanation is the presence of a younger hydrophobic contaminant. However, as many different peaks elute in this part of the chromatogram, identification will be difficult. The Gly-Pro-Hyp/Gly-Pro-Ala fraction is the oldest. If we roughly construct a mass/age balance by adding up the contribution of the hydrophobic fractions (the early fraction being by far the largest) and the Gly-Pro-Hyp/Gly-Pro-Ala fraction (the latter constituting about 20% of the mixture), we find that the age of the pooled fractions agrees with the age of the peptide mixture. Thus, we conclude that reversed-phase HPLC, using an organic solvent, such as acetonitrile, does not seri-

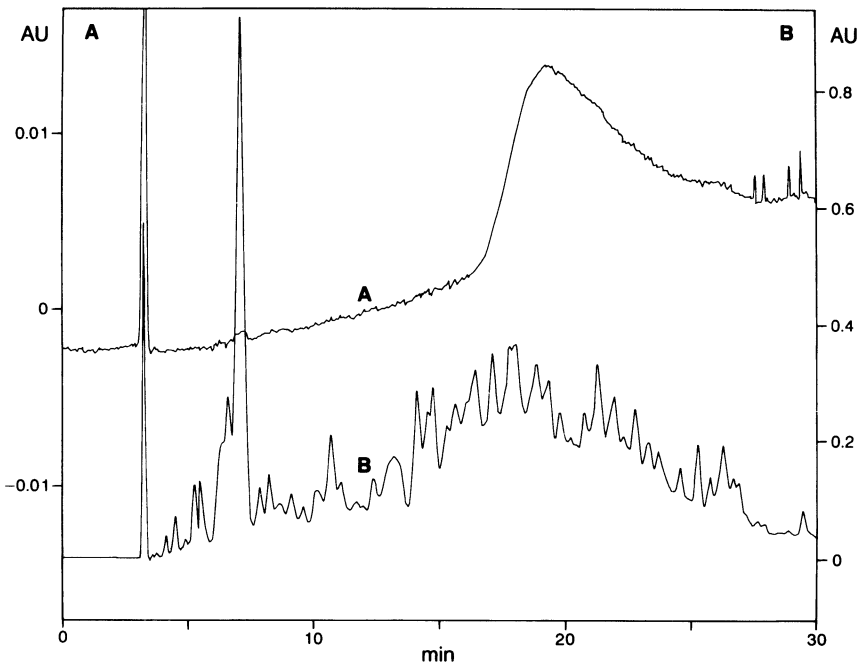

Fig. 2. Chromatogram of a preparative reversed-phase extraction of peptides. A. Detection at 320 nm, full-scale 0.02 absorbance units. B. 210 nm, full-scale 0.94 absorbance units. Gly-Pro-Hyp and Gly-Pro-Ala elute at about 7 min, the hydrophobic peptides between 12 and 30 min.

ously affect ^{14}C dating. There is a discrepancy between the GrN date and the "collagenase" dates, which should probably be attributed to laboratory contamination, but, given these first results, low ^{14}C backgrounds seem possible.

The Rise How sample is included because it had previously yielded ^{14}C dates of about 1300 BP, contradicting the expected Roman age (it was found under Roman watch towers near Hadrian's Wall in the UK). The acid-soluble fractions show the presence of contaminants of varying $\delta^{13}C$ and ^{14}C content. The spontaneous precipitate (which precipitated from solution *after* ultrafiltering) and the hydrophobic HPLC fraction differ somewhat from the peptide mixture and the Gly-Pro-Hyp/Gly-Pro-Ala fraction, in C/N ratio, $\delta^{13}C$ value and ^{14}C age. The latter two fractions show nearly identical ^{14}C ages, slightly younger than the ages obtained on amino-acid mixtures. We conclude that the discrepancy between expected and ^{14}C ages is more an archaeological than a dating problem.

The Stoney Island sample comes from an Irish bog body, and thus, may have humic contamination. Here also, we find large variations in the acid-soluble fractions, in C/N ratio, $\delta^{13}C$ and in ^{14}C content. The three peptide fractions obtained from the acid-insoluble fraction agree well with each other. These ages are somewhat higher than expected; however, similar early Neolithic bog bodies are known from Denmark.

From these first results, we can conclude that the use of both collagenase and of reversed-phase HPLC seems possible in ^{14}C chemistry, and that especially dating of the Gly-Pro-Hyp/Gly-Pro-Ala fractions from bone collagen seems to be quite reliable.

Future analyses will include quantification of digestion yields, ion-exchange HPLC of collagenase peptides and experiments to deglycosylate collagen prior to collagenase digestion using enzymes.

TABLE 1. Chemical, stable isotope and ^{14}C results on obtained fractions

Solubility in cold, weak acid	Fraction	C/N	%C	$\delta^{13}C$	^{14}C age	OxA no.
PCR 3, Groningen ^{14}C age: >46 ka BP, $\delta^{13}C$ = –20.76‰, GrN-13,000						
Insoluble:	Gelatin 1	3.1	21.5	–20.9		
	>10 kD gelatin 1	3.1	35.6	–22.3		
	<10 kD peptides from >10 kD gelatin 1	2.7	26.8	–20.1	30,800 ± 660	2946
	Gly-Pro-Hyp/Gly-Pro-Ala from above	2.7	21.7	–20.6	34,379 ± 1030	2947
	Hydrophobics early fraction from above	3.4	43.7	–22.5	29,100 ± 570	2948
	Hydrophobics late fraction from above	4.8	45.2	–36.0	21,460 ± 290	2949
Rise How, expected age: Roman						
Soluble:	>30 kD gelatin 2	2.3	0.7	–23.7	*	
	10 kD<x<30 kD fraction	6.7	5.5	–24.3	4440 ± 70	2954
	<10 kD fraction	14.2**	0.0	–31.8	1780 ± 70	2953
Insoluble:	Amino-acid mixture from gelatin 1				1330 ± 60	1818
	Amino-acid mixture from gelatin 1				1280 ± 60	1935
	Gelatin 1	3.3	12.9	–21.4		
	>10 kD gelatin 1	3.3	31.1	–20.2		
	>10 kD gelatin 1 spontaneous precipitate	3.8	45.0	–19.4	1670 ± 70	2955
	<10 kD peptides from >10 kD gelatin 1	3.2	37.3	–20.9	1205 ± 70	2936
	Gly-Pro-Hyp/Gly-Pro-Ala from above	2.9	38.8	–21.0	1180 ± 70	2937
	Hydrophobics from above	2.5	34.4	–22.6	190 ± 70	2938
Stoney Island, expected age: late prehistoric						
Soluble:	>30 kD gelatin 2	1.1	6.4	–24.3	*	
	10 kD<x<30 kD fraction	17.7**	9.7	–30.8	4035 ± 100	2939
	<10 kD peptides from >10 kD gelatin 2	28.6**	4.8	–20.4	101.9 ± 0.9%	2940
Insoluble:	Routine prep ion-exchanged gelatin 1			–22.6	6200 ± 80	2758
	Gelatin 1	3.9	41.6	–24.2		
	>10 kD gelatin 1	5.2	46.5	–24.0		
	<10 kD peptides from >10 kD gelatin 1	3.3	26.2	–21.7	5170 ± 90	2941
	Gly-Pro-Hyp/Gly-Pro-Ala from above	3.2	15.6	–21.3	5270 ± 80	2942
	Hydrophobics from above	3.3	42.1	–20.0	5180 ± 80	2943

*>modern. No values are given for these fractions because the results obtained indicate the presence of a significant (laboratory?) >modern contaminant. Some other samples in this batch undergoing the same treatment also were affected.
**Sample too small for reliable measurement

ACKNOWLEDGMENTS

We are especially grateful to Ms. Angela Bowles, who did much of the enzyme digestions, ultrafiltrations and combustions, and to all at the Radiocarbon Accelerator Unit involved in the ^{14}C measurements.

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