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Isolation of Amino Acids from Natural Samples Using Sublimation

Daniel P. Glavin and Jeffrey L. Bada*

Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093-0212

Amino acids have appreciable vapor pressures above 150 °C and will sublime under partial vacuum at elevated temperatures without any racemization or decomposition. The recoveries of several amino acids including aspartic acid, serine, glycine, alanine, α-aminoisobutyric acid, and valine were optimized by varying the temperature and duration of sublimation. Sublimation has been shown to be a rapid and effective technique for the isolation of amino acids from natural samples for enantiomeric analyses and a good substitute for conventional cation-exchange desalting techniques.

The standard procedure for isolating amino acids from natural samples involves both acid hydrolysis and desalting of the sample prior to analysis.[1,2] Both procedures require liquid reagents that can introduce laboratory contaminants to the sample. Acid hydrolysis requires heating the sample in double-distilled (2×) 6 N HCl at 100 °C for 24 h (hydrolysis for shorter periods at higher temperatures has also been used). The hydrolyzed sample is then desalted using cation-exchange chromatography, and amino acids are eluted from a column with ammonium hydroxide. Even with careful distillation and preparation of the reagents, blanks carried through the extraction procedure often contain significant background levels of L-amino acids, which compromise enantiomeric measurements of samples by reducing D/L ratios, especially for those samples with low amino acid contents.

Sublimation by itself requires no wet chemical reagents and eliminates the need for cation-exchange chromatography, a significant source of sample contamination. We have developed a "one-pot" procedure, in which sublimation is coupled together with a rapid and clean acid vapor hydrolysis technique, to isolate amino acids from natural samples such as mollusk shells and carbonate deep sea sediments. This process provides an efficient way for isolating amino acids from natural samples with minimal wet chemical workup. The sublimation-based isolation procedure could be especially useful in spacecraft instrumentation, where the use of corrosive liquid reagents required in traditional methods would be impractical. Future plans for in situ amino acid analyses on solar system bodies, including Mars, could use sublimation as a method for amino acid isolation.

EXPERIMENTAL SECTION

Chemicals. The free amino acids were commercial preparations from Sigma-Aldrich and consisted of L-alanine, glycine, α-aminoisobutyric acid (Aib) and racemic mixtures of aspartic acid, serine, and valine. Methylamine (MA), ethylamine (EA), and albumin were also obtained from Sigma-Aldrich. *N*-Acetyl-L-cysteine (NAC) and *o*-phthaldialdehyde (OPA) were reagent grade from Fisher Scientific. The H_2O and HCl were both doubly distilled, and NH_4OH was prepared by bubbling gaseous NH_3 through double-distilled water.

Apparatus and Methods. A sublimation apparatus (SA) has been designed to allow for acid hydrolysis, sublimation, and amino acid collection from natural samples (see Figure 1A). The SA consists of a quartz tube (2.5 × 31 cm) and a Pyrex glass coldfinger (1.6 × 15.5 cm) which are sealed together under vacuum with a clamp and O-ring. Quartz glass was necessary to ensure stability of the apparatus at high sublimation temperatures (up to 1100 °C). The coldfinger, insulated from the outside heat by a partial vacuum, is cooled to liquid nitrogen temperature (−195 °C). The sample is placed at the bottom of the SA and subjected to acid vapor hydrolysis by sealing a specially designed cap holding a few milliliters of 6 N HCl to the top of the SA (see Figure 1B) and heating the entire apparatus to 150 °C for 4 h.[3−5] After hydrolysis, samples are vacuum-dried at 250 mTorr and sealed prior to introduction into a temperature-controlled tube furnace (Lindburg Blue, maximum temperature 1150 °C). The naturally occurring temperature gradient inside the SA (furnace temperature to coldfinger temperature of −195 °C) facilitates the vaporization of amino acids at the bottom of the quartz tube and their condensation onto the end of the coldfinger. After sublimation is complete, the pressure inside the SA is equalized and the coldfinger washed with double-distilled water. Sublimed amino acids are then analyzed by OPA/NAC derivatization and HPLC separation.[6,7] The amino acid recovery was calculated by direct comparison with an amino acid standard.

Two powdered fossil Pleistocene mollusk shell samples[2] with ages of 50 kyr and 1 Myr and a 40-kyr carbonate-rich deep sea sediment[8] were used in the experiments. These samples were first sublimed inside the SA at 450 °C for 5 min without any

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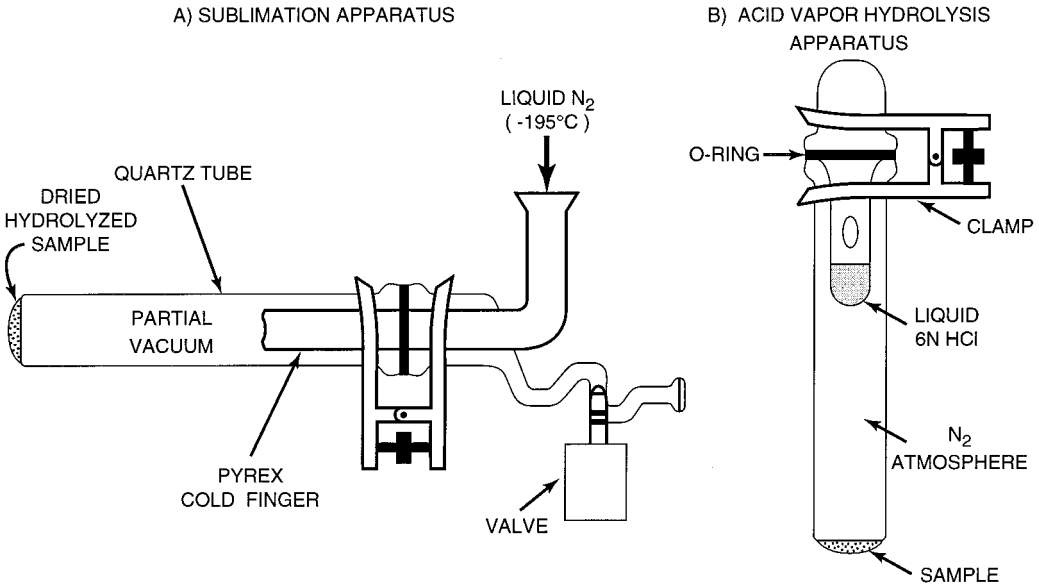

Figure 1. Diagram of the sublimation apparatus (A) and the vapor hydrolysis apparatus (B) used in the experiments (not drawn to scale).

preliminary acid treatment (untreated sublimed fraction). In addition, approximately 50 mg of each sample was sealed in the SA under nitrogen and acid vapor hydrolyzed as previously described. After vapor hydrolysis, the residues were partitioned into three additional extracts: one was analyzed directly by HPLC (undesalted fraction), a second was desalted using Bio-Rad AG50W-X8 cation-exchange resin prior to analysis (desalted fraction), and the remaining fraction was dried down in the SA, the SA evacuated to 250 mTorr, the sample sublimed at 450 °C for 1 min, and the sublimed residue then analyzed (sublimed fraction). The total amino acid concentrations as well as enantiomeric ratios for the various fractions were then determined.

RESULTS AND DISCUSSION

Free Amino Acids and Albumin. The stability of amino acids in aqueous solution at high temperatures and pressures has been studied extensively, and it has been shown that amino acids under these conditions are extremely unstable.[9,10] In an aqueous mixture of amino acids heated to 250 °C at 250 atm, the most predominant amino acid decomposition products were found to be amines formed by decarboxylation.[10] In addition, due to the presence of liquid water, amino acid racemization was also found to occur rapidly at these temperatures.

In contrast to previous reports on amino acid stability in solution, we found that, during sublimation, pure amino acids in a dry, low-pressure environment are extremely stable at high temperatures and do not decompose or racemize. Figure 2 shows the sublimation recoveries of aspartic acid, glycine, alanine, and valine at two different sublimation temperatures. Serine and Aib were also successfully sublimed at both temperatures, although serine recoveries were much lower and similar to aspartic acid. The vapor pressures of the amino acids in Figure 2 have been reported to be between 200 and 450 mTorr at 200 °C,[11] and thus

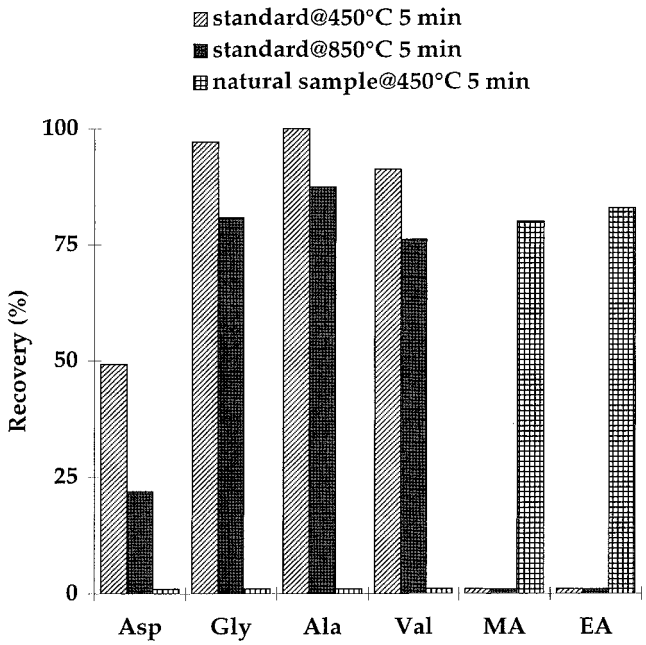

Figure 2. Results of sublimation experiments using a standard amino acid mixture containing free amino acids (aspartic acid, glycine, alanine, valine) and a natural sample containing a similar suite of protein amino acids. For the standard amino acids, no detectable decomposition into methylamine (MA) or ethylamine (EA) was found at any sublimation temperature. In the case of the natural samples, only trace amounts of sublimed amino acids were detected, but high recoveries of their amine decomposition products were found.

at an internal SA pressure of 250 mTorr, we were able to recover a large fraction of these amino acids by sublimation at temperatures above 150 °C. Furthermore, we found that there was no racemization of aspartic acid, alanine, or valine (D+L serine could not be separated with our HPLC method) at any temperature as these amino acids were heated in the absence of liquid water. The preservation of enantiomeric ratios during sublimation is consistent with earlier experiments by Gross and Grodesky,[12] who observed no change in the optical activity of sublimed L-amino

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acids. The low recoveries of aspartic acid and serine (10−50%), especially at lower temperatures, is due to their sluggish response to temperature.[12] At high temperatures, amino acid sublimation was found to be very rapid as 80−100% of the amino acids (except aspartic acid and serine) could be recovered after 30 s at 450 °C and after just 5 s at 1100 °C. These surprising results demonstrate that at higher temperatures amino acids respond very rapidly to the heat, subliming intact onto the coldfinger within seconds after the SA is placed in the furnace. The rapid sublimation occurs 10−100 times faster than originally estimated by Gross and Grodesky and is consistent with heat-transfer calculations which predict that temperature equilibration between the furnace and the inside of the SA should take less than 3 s. The optimal recovery of the standard was obtained at 450 °C for 1 min where recoveries of 90−100%, except for aspartic and serine, were found for all of the tested amino acids.

Polymerization of amino acids, resulting in the formation of dipeptides and diketopiperazines (i.e., cyclic dipeptides), has been observed in the vapor state.[13] Basiuk et al. suggested that, during sublimation, amino acid peptide formation can occur in the vapor state on the surface of both aluminum and silica.[13] However, we acid hydrolyzed the sublimed amino acids and did not detect an increase in overall amino acid recovery. Thus, we found no evidence for the formation of either peptides or diketopiperazines in any of our sublimation experiments with free amino acids.

We also sublimed pure albumin (isolated from chicken egg) at 450 °C for 5 min and found that although some (1−10%) of the protein-bound amino acids were sublimed and recovered intact, a much larger fraction was destroyed, which suggests that peptide bonds may inhibit amino acid sublimation. No amines were detected after sublimation, which indicates that amino acid decarboxylation did not occur when this protein was heated. After the albumin was acid vapor hydrolyzed, 80−100% of the amino acids could then be recovered from the protein after sublimation at 450 °C for 5 min.

Natural Carbonate Samples. Because high concentrations of amino acids are present in the organic matter associated with natural carbonate shells and sediments,[2,14,15] this type of sample was used to investigate whether sublimation could be used to isolate amino acids from natural samples. The sublimation of amino acids from natural samples was found to be more difficult than with pure amino acid mixtures (see Figure 2). For the standard free amino acids and albumin, no decomposition into amines was found during sublimation at any temperature. Unlike the pure amino acid mixtures, amino acids inside natural carbonate samples were not readily sublimed at 450 °C and, instead, underwent decarboxylation into amines at this temperature. Above 450 °C, virtually all of the amino acids originally present in the carbonate samples were destroyed by thermal heating. The most predominant decomposition products in these samples after sublimation at 450 °C for 5 min were methylamine and ethylamine, produced by the decarboxylation of glycine and alanine, respectively. We found that, after sublimation, 80% of the glycine and alanine originally present in the samples decomposed into amines. Ammonia was also detected in some of the experiments, and it can be attributed to deamination of some of the amino acids. We observed an increased production of both ammonia and amines with increased time and temperature of sublimation.

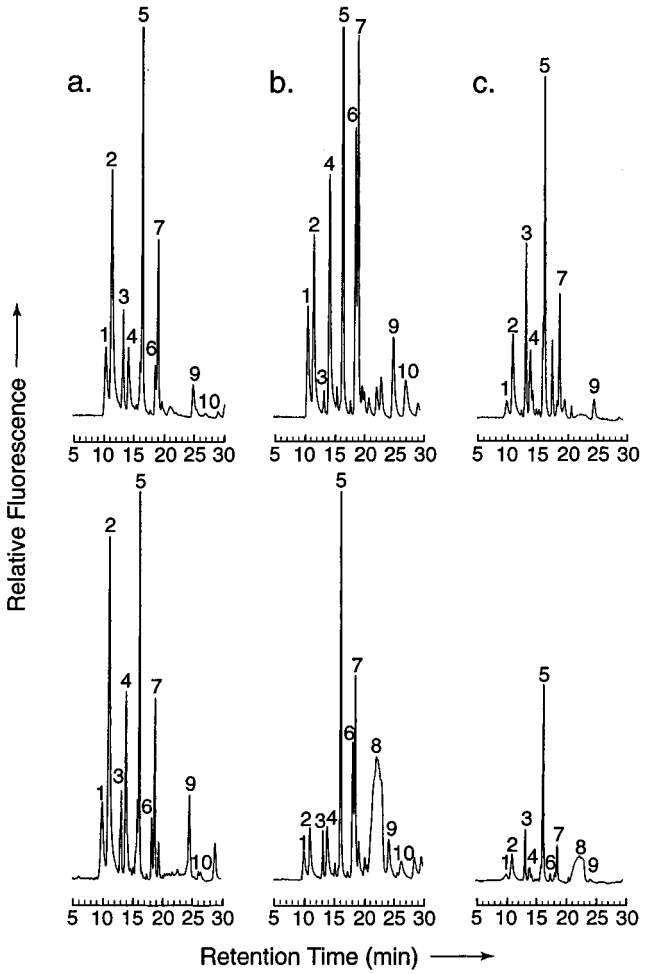

Figure 3. The 5−30-min reversed-phase HPLC chromatograms of the HCl vapor hydrolyzed, desalted (top) and sublimed (bottom) extracts from a 50-kyr mollusk shell (a), a 1-Myr mollusk shell (b), and a 40-kyr deep sea sediment (c). Peaks were identified by comparison of the retention times of an amino acid standard run at the same time. Peak identifications: (1) D-aspartic acid; (2) L-aspartic acid; (3) D/L-serine; (4) D/L-glutamic acid; (5) glycine; (6) D-alanine; (7) L-alanine; (8) ammonia; (9) L-valine; and (10) D-valine.

Minerals in natural shells and sediments such as calcium carbonate provide sites by which amino acids can form strong ionic bonds with the matrix,[16−18] thereby preventing the sublimation of amino acids. In addition, because in the mollusk shells a portion of the amino acids are peptide bound, which do not sublime as readily as free amino acids as demonstrated with albumin, amino acid decomposition occurred when these samples were heated.

We found that amino acids could only be liberated and sublimed intact from the mollusk shell and deep sea sediment

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Table 1. Summary of the Recovery of Amino Acids from Natural Samples in Desalted and Sublimed Extracts[a]

	aspartic acid		alanine		valine	
	rec	D/L	rec	D/L	rec	D/L
Fossil Shells						
5×10^4 years old						
desalted	146	0.46	35	0.45	23	0.17
sublimed	6−22	0.45	2−22	0.39	1−17	0.19
Wehmiller		0.38		0.36		0.16
10^6 years old						
desalted	37	0.98	31	0.89	16	0.80
sublimed	5	0.96	5	0.99	5	0.82
Wehmiller		0.88		0.95		0.85
Deep Sea Sediment						
4×10^4 years old						
desalted	27	0.32	11	0.23	18	<0.05
sublimed	1	0.33	0.3	0.25	0.1	<0.04

[a] All samples were HCl vapor hydrolyzed at 150 °C for 4 h. The amino acid recoveries for each method are given in parts per million (ppm). The D/L ratios have been corrected using a racemic standard and are therefore different from the uncorrected peak height ratios shown in Figure 3. The uncertainties of the measurements are ±1 ppm for the recoveries and ±0.03 for the enantiomeric ratios. Also shown are the D/L ratios reported by Wehmiller[2] for the fossil shells.

samples after the carbonate matrix had been dissolved and the peptide bonds hydrolyzed with hot 6 N HCl vapor. Following HCl vapor hydrolysis, the residue was sublimed in the SA under the conditions of optimum amino acid recovery previously determined (450 °C for 1 min at 250 mTorr). Several amino acids isolated from the samples using traditional desalting methods (Figure 3, top), including aspartic acid, serine, glycine, alanine, and valine, were also recovered from the mollusk shells and deep sea sediment following acid hydrolysis and sublimation (Figure 3, bottom). Although the recovery of the amino acids by sublimation was lower than that obtained from the desalted extracts (top), no amino acid racemization was detected after sublimation and thus enantiomeric ratios were preserved. Analyses of the undesalted hydrolyzed extracts that were not sublimed gave inconsistent results and D/L values different from reported ratios.

In Table 1 we report the calculated amino acid recoveries and D/L ratios for aspartic acid, alanine, and valine in the desalted and sublimed extracts. The sublimation method yields amino acid contents lower than those determined using the traditional desalting method. However, the sublimed amino acid D/L ratios are consistent with the ratios determined in the desalted extracts as well as with the values reported by Wehmiller.[2] There was some ammonia detected in the sublimed extracts of the 1-Myr mollusk shell and the 40-kyr deep sea sediment (broad peak at ~20 min), which could inhibit derivatization, hence the lower yields relative to the 50-kyr shell sample.

CONCLUSIONS

The sublimation and successful recovery of pure amino acids at reduced pressures and high temperatures has been demonstrated. Subliming intact amino acids from albumin, carbonate shells, and deep sea sediment without decomposition was also achieved, although an acid vapor hydrolysis treatment of the samples prior to sublimation was necessary. In traditional amino acid analyses, hydrolyzed samples must first be desalted before HPLC analysis as salts interfere with the OPA/NAC derivitization reaction and greatly reduce the lifetime of HPLC columns. Our rapid sublimation procedure eliminates the need for cation-exchange chromatography and desalting altogether as sublimed samples can be derivatized directly and analyzed for the presence of amino acids without the need for wet chemical extraction methods. Although the total recovery of sublimed amino acids from the natural samples was not as high as the recovery obtained from desalted samples, D/L enantiomeric ratios were preserved. Amino acid enantiomer analyses can be carried out using this "one-pot" hydrolysis/sublimation procedure in less than 5 h compared to the traditional method which requires more than 1 day for amino acid isolation and analyses. We are continuing investigations on how to further optimize the recovery of amino acids and other organic compounds from a variety of natural samples using this sublimation-based method.

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