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Decomposition of Aspartame Caused by Heat in the Acidified and Dried State

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L-Aspartyl-L-phenylalanine methyl ester (aspartame) forms a variety of degradation products when heated in an acidified–lyophilized state at 110 °C for 24 h in vacuo. In addition to the well-characterized degradation products of aspartame, our results suggest that aspartame can also dehydrate upon heating and that oligopeptides can be generated containing higher ratios of aspartic acid to phenylalanine than the starting material. Aspartame dehydrates to form an anhydro derivative (MW 276) as determined by fast atom bombardment mass spectrometry. Photoacoustic infrared spectroscopy results suggest an anhydride can occur between the β-carboxyl group and the peptide linkage upon heating.

L-Aspartyl-L-phenylalanine methyl ester (aspartame) is used today widely as an artificial sweetener in a variety of food products and beverages, but it is not commonly used as an additive in cooking or baking because of its instability at high temperatures (FDA, 1981). Decomposition products are known to form by heating aspartame in solution or in the dry state. In solution, its stability depends on the pH and temperature (Holmer, 1984; Harper, 1975; Mazur, 1976; Furda et al., 1975; Prudel et al., 1986). A diketopiperazine and products derived from hydrolysis of the peptide and ester bonds have been identified. Recently, it has been shown that extensive racemization of bound aspartic acid occurs at 100 °C in a couple of days at pH 7.0 in the diketopiperazine product (Boehm and Bada, 1984). At pH 4.0, less racemization takes place, and it occurs in intact aspartame. In the dry state, the stability of aspartame is considered reasonably good. Mazur (1976) states that only 5% of the diketopiperazine is formed after heating for 70 h at 105 °C.

In a recent report, Luo et al. (1987) found that aspartyl- and asparaginylglycine-containing peptides are sensitive to heat in the dry state. A cyclic imide, proven by fast atom bombardment mass spectrometry and Fourier transform infrared photoacoustic spectroscopy, occurs when peptides are acidified, lyophilized, and then heated in vacuo. Because of these effects, we decided to examine further the stability of aspartame in the dry state. The results reported herein show that aspartame is not very stable at 90 °C or above if it is acidified and lyophilized before heating. A number of new products are formed and identified by HPLC, amino acid analysis, FAB–mass spectrometry, and photoacoustic infrared spectroscopy. An anhydride containing aspartic acid and phenylalanine methyl ester is suggested to form upon heating. Peptide products with higher ratios of aspartic acid to phenylalanine than aspartame are indicated.

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MATERIALS AND METHODS

L-Aspartyl-L-phenylalanine methyl ester (aspartame) was purchased from Sigma Co. All other chemicals and reagents were of high-quality analytical grades.

Heating Procedures. Aspartame was dissolved in distilled water to a concentration of 5 mg/mL. Aliquots of this solution were adjusted to pH 2.0, 3.0, or 7.0 with 0.1 M HCl or 0.1 M ammonium hydroxide. Each aliquot was freeze-dried in a hydrolysis tube (Pierce Chemical Co.). The tubes were sealed under vacuum and heated at various temperatures for 24 h.

Paper Electrophoresis. The heated aspartame was analyzed by high-voltage paper electrophoresis. Sample (1–2 μL) was applied to the middle of a 20-cm electrophoresis paper. Electrophoresis was performed in a buffer of 2.4% pyridine and 0.6% acetic acid (pH 5.6), in water at 2000 V for 1 h. Aspartame and thermal degradation products were identified by reaction with fluorescamine.

HPLC Separation. Degradation products were separated on a Microsorb C_{18} reversed-phase HPLC column (4.6-mm i.d. × 25-cm length, Rainin Instruments Co.). Buffer A consisted of 0.1% trifluoroacetic acid in water. Buffer B was 90% acetonitrile, 10% water, and 0.1% trifluoracetic acid. Elution was performed at 1 mL/min with a 38-min linear gradient of 0–60% buffer B, and the products were detected at 260 nm, collected, pooled, and lyophilized to remove solvent.

FTIR-PAS. Fourier transform infrared photoacoustic spectroscopy (FTIR-PAS) is useful for determining the structure of peptides and proteins (Renugopalakrishnan and Bhatnagar, 1984). In this study, all IR spectra were measured with a Perkin-Elmer Model 1800 FTIR spectrometer equipped with a METC Model 100 photoacoustic cell. Sample spectra were normalized by dividing the sample spectrum by a carbon black spectrum.

Aspartame and its degradation products were applied on a thin layer of collodion membrane as a water solution.

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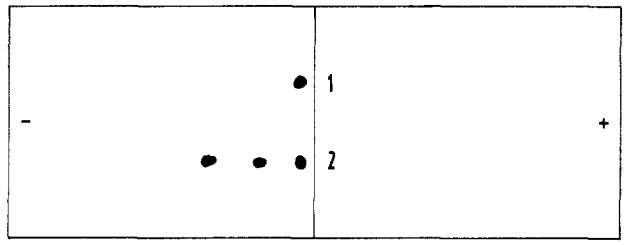


Figure 1. Electrophoretogram of aspartame heated at 110 °C for 24 h: in vacuo (lane 1); in vacuo after being acidified to pH 2 and lyophilized (lane 2). Materials were detected by reaction with fluorescamine and observance of fluorescence utilizing UV light.

After drying, the peptide formed a thin-layer solid sample on the membrane. The spectrum of collodion membrane was removed from the peptide spectrum by spectral subtraction.

The IR spectra of succinimide, succinic anhydride, and glycylglycine ethyl ester were taken under the same conditions for comparison.

Amino Acid Analysis. Some degradation products were analyzed by amino acid analysis. All amino acid analyses were performed on a Durrum 400 analyzer. The HPLC fractions were hydrolyzed at 110 °C for 24 h in constant-boiling HCl with redistilled phenol (0.1%) added. To determine whether any free aspartic acid was present, some samples were diluted with buffer solution (pH 2.2) and injected directly into the analyzer without hydrolysis. Elution was performed with pH 3.15 sodium citrate buffer (0.067 M in Na^+) for 18 min followed by pH 4.03 sodium citrate buffer (0.1 M in Na^+) for 39 min and pH 5.15 sodium citrate buffer (1.4 M in Na^+) for 45 min. Amino acids were quantified with respect to an amino acid standard (Standard H from Pierce Chemical Co.).

Fast Atom Bombardment Mass Spectrometry. Mass spectra were obtained on a Kratos MS 50 TC double-focusing mass spectrometer with DS 90 data system, operating at 5–8-kV accelerating voltages. Aspartame and its degradation products were dissolved in glycerol or magic bullet (dithioerythritol/dithiothreitol mixture); 1–2 μL of the sample/matrix mixture was deposited on the end of the copper FAB tip. In all cases, the reference spectrum of the matrix was subtracted.

RESULTS

When a solution of aspartame at neutral pH is lyophilized and the resulting residue is heated in vacuo at 110 °C for 24 h, redissolved in water, and examined by high-voltage electrophoresis, only one electrophoretically neutral and fluorescamine-positive species can be identified (Figure 1, lane 1). The migration corresponds to that of unheated aspartame, and the results are consistent with the earlier statement of Mazur (1976) about the stability of aspartame in the dry state. If a solution of aspartame is acidified to pH 2.0 or 3.0 and the process is repeated, the results obtained are quite different. Figure 1, lane 2, is a sketch of an electrophoretogram and shows that, in addition to neutral material, electrophoretically positively charged substances can be identified. Very little, if any at all, negatively charged material is found by this procedure.

To determine the effect of temperature on aspartame in the acidified and dried state, samples were heated at various temperatures for 24 h and analyzed by HPLC. Figure 2A shows the elution profile of the starting aspartame. Samples heated at 45, 58, and 72 °C showed little or no change. A sample heated at 90 °C showed some new products and a splitting of the main peak. At 110 °C, the

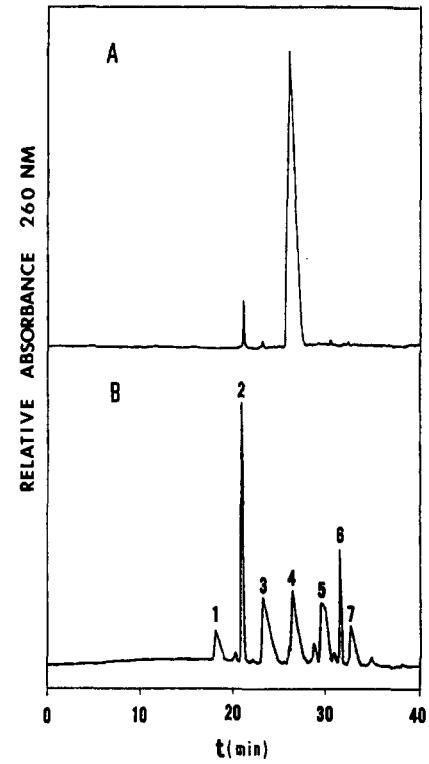


Figure 2. HPLC separation of aspartame and its degradation products: (A) elution profile of the starting aspartame; (B) separation profile of degradation products of aspartame. Aspartame was heated at 110 °C for 24 h in vacuo after being acidified to pH 2.0 and lyophilized.

degradation is extensive as shown by the profile in Figure 2B. To elucidate what these fractions represent, the material was collected and subjected to further analysis. Peaks 1 and 3 contain only phenylalanine as determined by amino acid analysis after acid hydrolysis. Peak 3 corresponds to the most positively charged migrating material found in the electrophoresis experiment of Figure 1, so we presume that peak 3 is phenylalanine methyl ester. Peak 1 probably is free phenylalanine on the basis of amino acid analysis and that it is electrophoretically neutral. Peak 2 is suggested to be the diketopiperzine because it contains equivalent amounts of aspartic acid and phenylalanine and does not react with fluorescamine. Peak 4 also contains equivalent amounts of the two amino acids but shows both neutral and acidic material on high-voltage electrophoresis. The neutral material could be the starting aspartame because it has a retention time nearly identical with that of aspartame. The acidic material could represent free dipeptide. Peak 5 contains nearly equivalent amounts of aspartic acid to phenylalanine (1.3/1), but peak 7 contains more than 2 equiv of aspartic acid to phenylalanine (2.2/1). No free amino acids are found in peaks 5 and 7 before acid hydrolysis. Peaks 5 and 7 migrate as positively charged fluorescamine-staining materials on high-voltage electrophoresis similar to that shown in the middle section of the electrophoretogram of Figure 1 for heated aspartame. A small amount of neutral material is also seen in these fractions. No information is available about peak 6 because of the limited material obtained.

Because material in peaks 5 and 7 had characteristics quite different from other degradation products of aspartame previously reported, materials from these fractions were subjected to FAB–mass spectrometry. One possibility that could explain the positively charged material is a loss of the negative charge of the carboxylate group of aspartic acid through a cyclization reaction. An imide or an an-

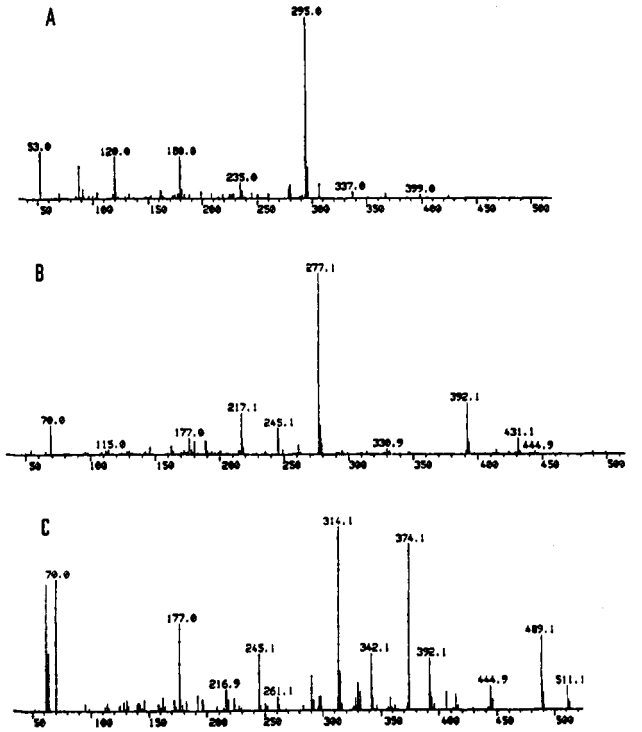


Figure 3. FAB–mass spectra of aspartame and degradation products: (A) aspartame; (B) degradation product of aspartame in peak 5 of Figure 2; (C) degradation product of aspartame in peak 7 of Figure 2.

hydride could form, respectively, by reaction of the amide N or the carbonyl O with the β-carboxyl group. Both products are dehydrated forms of aspartame. Figure 3A shows the fragmentation of aspartame. The protonated molecular ion $(M + H)^+$ of 295 is expected, and the other main fragments are anticipated from known cleavages of peptides in FAB–mass spectrometry; e.g., the peak at 235 is an alkyl cation formed from the loss of the $C(O)OCH_3$ function from aspartame (MW 294), the 180 peak represents a protonated form of phenylalanine methyl ester, and the 120 peak is $^+NH_2{=}CHCH_2Ph$. Figure 3B shows the pattern obtained of peak 5. The principal species is a quasi-molecular ion $(M + H)^+$ at m/z 277. This species differs from aspartame by 18 mu and presumably is a dehydrated form. Notice also that a small amount of a species with a mass of 392 is present. Its significance will be discussed later. The 245 form represents a loss of methanol from the 277 form, and the 217 species represents a loss of a $C(O)OCH_3$ function. Higher molecular weight species are found in peak 7, including a prominent component at 489 (Figure 3C). Losses of fragments from the 374 form identical with that seen for peak 5 can explain the presence of the 342 and 314 species, respectively. Higher molecular weight species are found in peak 7. Similar losses of methanol and $C(O)OCH_3$ from the carboxy terminal end of the peptide (374) can explain the 342 and 314 peaks, respectively.

To determine whether the material in peak 5, a dehydrated form of aspartame, has the characteristics of an imide or an anhydride, photoacoustic infrared spectroscopy was done. The FTIR-PAS spectra of aspartame and its degradation product (peak 5) are presented in Figure 4. B is a spectrum of aspartame. The strong amide A band at 3319 cm^{-1} is indicative of an NH···O hydrogen bond (Susi, 1972; Renugopalakrishan and Bhatnagar, 1984). The amide II band is also very sharp and is located at 1544 cm^{-1}. The amide II vibration is a mixed mode consisting of C=O stretching and N—H bending. Therefore, these

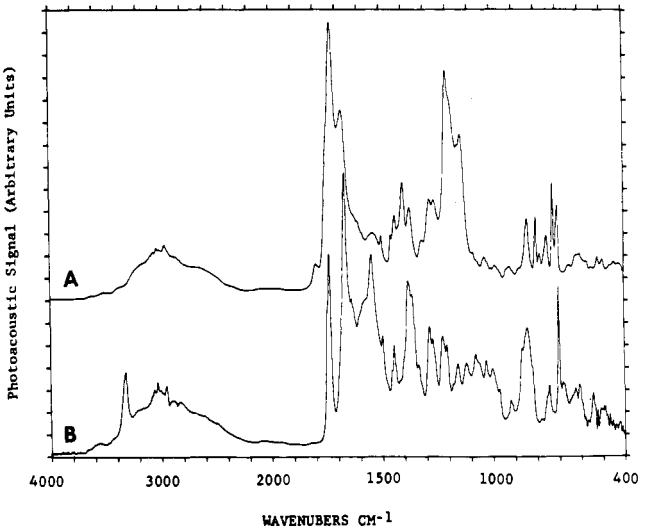


Figure 4. FTIR-PAS spectra of aspartame and degradation product: (A) degradation production peak 5 of Figure 2; (B) aspartame.

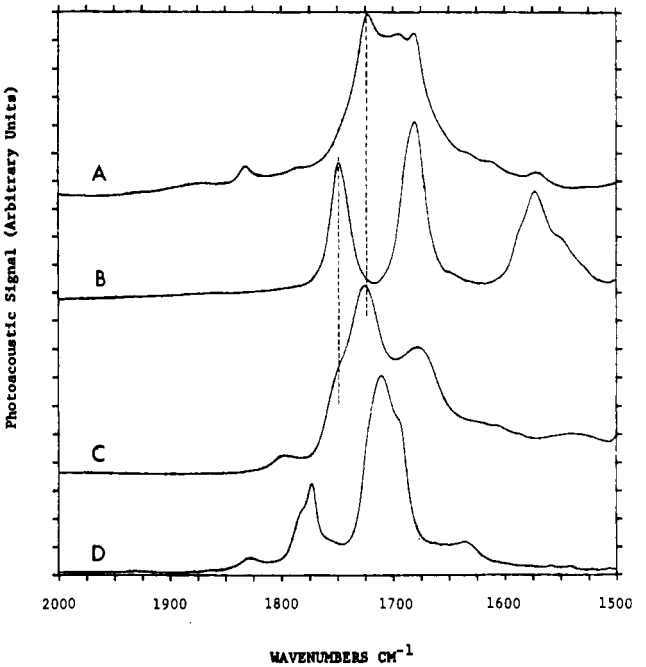
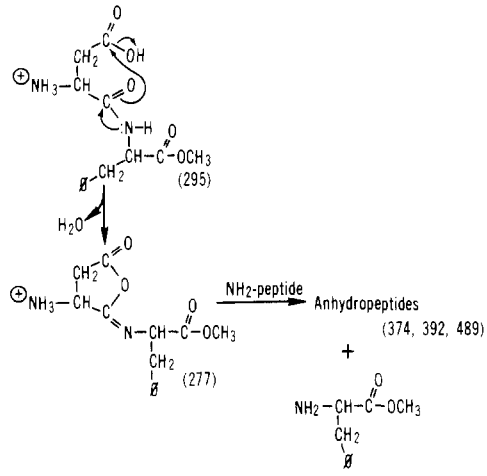


Figure 5. FTIR-PAS spectra of succinic anhydride (A) and glycylglycine ethyl ester (B) and degradation product of aspartame in peak 5 of Figure 2 (C) and succinimide (D).

two bands are related to the peptide bond in aspartame. However, in spectrum A, the degradation product, both amide A and amide II bands are absent and strongly suggest that the peptide bond in aspartame is involved in a cyclization reaction that occurs during heating. The hydrogen, which is linked to the nitrogen in the peptide bond, is missing after heating causing the loss of amide A and amide II bands.

The FTIR-PAS spectra of succinic anhydride, glycylglycine ethyl ester, degradation product (peak 5) of aspartame, and succinimide are shown in Figure 5. It is well-known that carbonyl compounds give rise to a strong band at 1900–1550 cm^{-1} caused by the stretching of the C=O bond. In the spectrum of succinic anhydride (A), the 1723-cm^{-1} peak can be assigned to C=O stretching, although it has shifted to a lower position than observed in conventional IR (1750 cm^{-1}). In spectrum B, the peak at 1748 cm^{-1} arises from the ester carbonyl stretching, and the peaks at 1680 and 1573 cm^{-1} are amide I and amide

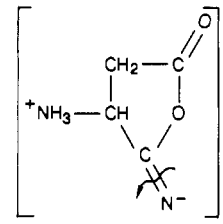
Scheme I



II, respectively. There are two peaks in the spectrum of succinimide, the intense peak is at 1710 cm^{-1}, and the other is at 1780 cm^{-1}, and they can all be assigned to C=O stretching. If we compare the spectrum of degradation product (peak 5) with these spectra, the strong peak at 1725 cm^{-1} is just at the same position as the intense peak of succinic anhydride. There is a shoulder at about 1750 cm^{-1}; it can be assigned to the ester carbonyl stretching and coincides with that observed in the spectrum of glycylglycine ethyl ester. There is another weaker peak at 1678 cm^{-1}; it may be due to C=N stretching. Colthup et al. (1975) have summarized the spectral regions for the C=N group; absorbances at 1690–1645 cm^{-1} are for the OC=N group. A C=N stretching of an OC=N linkage of an anhydride could explain our results. Because of the similarities with succinic anhydride and no common features with succinimide, it is suggested that material in peak 5 is related to an anhydride.

DISCUSSION

Our results suggest that aspartame can dehydrate to form an anhydride-containing species when it is heated in the acidified and dried state. A mechanism that can account for the formation of the anhydride and derived products is illustrated in Scheme I. The instability of aspartame under acidified conditions is attributed to protonation of the β-carboxyl group. Without protonation, the O^- group of a carboxylate ion is not a good leaving group and hence could explain why cyclization as depicted is not seen under neutral conditions. Similar results were found for cyclization of aspartyl glycine containing peptides by Luo et al. (1987). The mass spectrometry results show that a 277 species is formed, and the photoacoustic infrared spectroscopy shows that this species has the characteristics of an anhydride, but not an imide. The electrophoretic properties (positive charge) and amino acid analysis results are consistent with a proposed structure for the 277 species. Mass spectrometry and amino acid analysis of the HPLC fraction (peak 7) showed that higher molecular weight forms are produced upon heating and that these structures contain more aspartyl equivalents than phenylalanine equivalents. The combination of an aspartyl unit



with aspartame and concomitant loss of hydrogen could account for the formation of an anhydropeptide, the 392 species. Its reaction with the 277 and 392 species accounts for the formation of the 374 and 489 species, respectively. On the basis of the results of amino acid analyses we suggest that peak 5 (aspartic acid/phenylalanine = 1.3/1) contains mainly an anhydride between aspartic acid and phenylalanine with a small amount of material containing 2 equiv of aspartic acid and phenylalanine (392 form). Peak 7 (aspartic acid/phenylalanine = 2.2/1) contains as its major fraction an anhydrotripeptide (374 form) and smaller amounts of a 392 species and a tetrapeptide derivative (489 form).

The studies show that aspartame is unstable when it is heated in an acidified and dried state. In addition to the well-described degradation products reported earlier, new decomposition products have been identified and partially characterized. Work is in progress to establish the exact nature of these new products.

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