traditional and semimodern mills unsuitable for use as a feed and for oil extraction. The results of the study also indicate that even though parboiling results in a high oil content in rice bran, the use of parboiled bran for oil extraction will be advantageous only if it is obtained from a modern type of rice mill where contamination of bran with husk could be minimized.

Registry No. Starch, 9005-25-8.

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Determination of Aspartame and Its Breakdown Products in Soft Drinks by Reverse-Phase Chromatography with UV Detection

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A rapid and simple analytical method is presented for the determination of aspartame and its breakdown products in carbonated soft drinks which had been stored at 22 ± 1 °C for various periods. The samples were analyzed by high-performance liquid chromatography (HPLC) with a reverse-phase column and UV detection at 214 nm under isocratic conditions. Four breakdown products were identified and their relative proportions determined. No aspartame condensation product, such as benzaldehyde–aspartame, was observed. The data showed that aspartame is relatively unstable in carbonated soft drinks over extended periods of storage, and that the breakdown products from hydrolysis and cyclization accounted for the aspartame lost.

INTRODUCTION

Aspartame (*N*-L-α-aspartyl-L-phenylalanine methyl ester), a high potency noncarbohydrate sweetener, is currently used in tabletop sweeteners, chewing gums, breakfast cereals, and carbonated soft drinks as well as powdered drinks and dessert mixes in the United States. It is claimed to have a sweetness profile similar to that of sucrose, which should give it a subtantial advantage over saccharin (Cloninger and Baldwin , 1974; O'Sullivan, 1983; Stegink and Filer, 1984).

Almost every major soft drink manufacturer in the United States now uses both aspartame and saccharin, usually in combination, to sweeten diet drinks. The blending of these two noncarbohydrate sweeteners serves to hold down the cost and helps to prolong the shelf life of the beverages, against the cost and shelf life of aspartame alone. Aspartame hydrolyzes slowly in the low pH range used in soft drinks, so products containing exclusively aspartame become less sweet on prolonged storage (Homler, 1984).

Aspartame is the methyl ester of the dipeptide aspartyl phenylalanine and under a wide range of stressful conditions (heat, moisture, and pH) may be susceptible to degradation. The stability of this high potency sweetener in various media was reported recently by Homler (1984). This author showed that aspartame-based cola, stored at 20 °C for 6 months, remained acceptably sweet relative to saccharin-sweetened beverages. The levels of aspartame left in the carbonated beverages and its breakdown and condensation products, however, were not reported. A recent study by Hussein and co-workers (1984) showed that aspartame is quite reactive with aldehydes—which are the principal flavor compounds used in chewing gums and in some soft drinks.

HPLC methods for the determination of aspartame have been reported (Argoudelis, 1984; Cross and Cunico, 1984; Daniels et al., 1984; Hussein et al., 1984; Scherz et al., 1983; Tyler, 1984; Webb and Beckman, 1984). These methods allowed the determination of aspartame to be made in the presence of other additives in beverages such as saccharin, caffeine, and sodium benzoate. No attempts, however, have been made by the above authors other than Scherz to identify any of the hydrolysis or degradation products of aspartame. Scherz and co-workers (1983) developed a reverse-phase HPLC method using gradient elution to separate aspartame, aspartylphenylalanine (AP), and 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP).

In this paper, the effects of storage on aspartame are investigated in soft drinks after standing at 22 ± 1 °C for varying periods of time. Soft drinks were analyzed for aspartame and its breakdown products with a simple isocratic HPLC procedure. This work is particularly relevant because of the growth in use of this new noncarbohydrate sweetener in carbonated soft drinks.

EXPERIMENTAL SECTION

Chemicals and Reagents. Organic-free water was obtained from a Milli-R/Q water purifier (Millipore Co., Bedford, MA). LC quality acetonitrile (Burdick & Jackson

Sugar Processing Research, Inc., Southern Regional Research Center, New Orleans, Louisiana 70179 (W.-S.T. and M.A.C.), and U. S. Department of Agriculture, ARS, Southern Regional Research Center, New Orleans, Louisiana 70179 (F.W.P.).

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Lab., Muskegan, MI) was used. Monobasic potassium phosphate (KH_2PO_4) was Baker analyzed grade (J.T. Baker). L-Aspartyl-L-phenylalanine methyl ester, L-phenylalanine methyl ester hydrochloride, L-phenylalanine, sodium saccharin, and caffeine were obtained from Aldrich Chemical Co. (Milwaukee, WI).

5-Benzyl-3,6-dioxo-2-piperazineacetic acid (DKP) was synthesized by heating solid aspartame (APM) at 150 °C in an oil bath overnight (Mazur, 1983). Aspartylphenylalanine (AP, 63%) was obtained along with DKP (37%) from base-catalyzed hydrolysis of aspartame.

Samples. It was decided to limit the study to diet drinks made in Canada which are sweetened exclusively with aspartame. Diet drinks have been made in the U. S. with aspartame only since 1983, whereas aspartame-sweetened drinks have been available in Canada for several years. The U. S. diet drinks generally contain a mixture of aspartame and saccharin. The choice of Canadian beverages eliminated the large saccharin peak which would otherwise interfere with the detection of the degradation products. Furthermore, the exact amount of aspartame added to the soft drinks is indicated on the can label in Canadian beverages.

The soft drink samples, made and purchased in Canada, were kindly supplied by the Sugar Association, Inc., Washington, D.C. Four different brands of aspartame-based soft drinks were used for analysis of the content of aspartame and its degradation products after various storage periods. The carbonated beverages were degassed in a sonic bath and then injected directly into the chromatographic system after filtration through a 0.45-μm Millipore filter.

HPLC. Analyses were carried out with a Waters Associates Model 200 liquid chromatograph equipped with a Model 6000A pump and an extended wavelength module (214 nm) (Waters Associates, Milford, MA). The samples were injected by Waters' WISP 710A sample autoprocessor. The column was Waters' μBondapak C18 reverse-phase column (3.9 mm × 30 cm) operated at ambient temperature. Chromatograms were displayed on a Linear Model 500 recorder (Linear Instruments Corp., Hackensack, NJ 07601). Data were stored and analyzed by an in-house LAS computer system (HP 3357). The chart speed of the recorder was $^1/_3$ cm/min. The detector was set at 214 nm on a range of 0.2 AUFS. The flow rate was 0.8 mL/min. Each soft drink sample was assayed in duplicate and the injection volume was generally 6 μL.

Solvents. The mobile phase used was acetonitrile and 0.0125 M KH_2PO_4 buffer (10:90) at 0.8 mL/min. The phosphate buffer was made up of 0.17% potassium phosphate solution adjusted to pH 3.5 with phosphoric acid.

Standard Solutions. Each standard was accurately weighed, transferred into separate 100-mL volumetric flasks, and diluted to volume with water. Standards: (1) 40.00 mg of aspartame (APM, 0.04%), (2) 30.00 mg of 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP, 0.03%), (3) 30.00 mg of phenylalanine (PA, 0.03%), (4) 10.80 mg of L-phenylalanine methyl ester hydrochloride to give a 0.009% L-phenylalanine methyl ester (PM) solution.

Aspartylphenylalanine (AP) standard was prepared by dissolving 50.00 mg of APM in 60 mL of water. The solution was adjusted to pH 8 with diluted sodium hydroxide and then made up to 100 ml with water. After standing for one day, DKP and AP were formed in a ratio of about 37:63 while no APM remained in the solution. The amount of aspartylphenylalanine formed is deduced from the difference between APM and DKP.

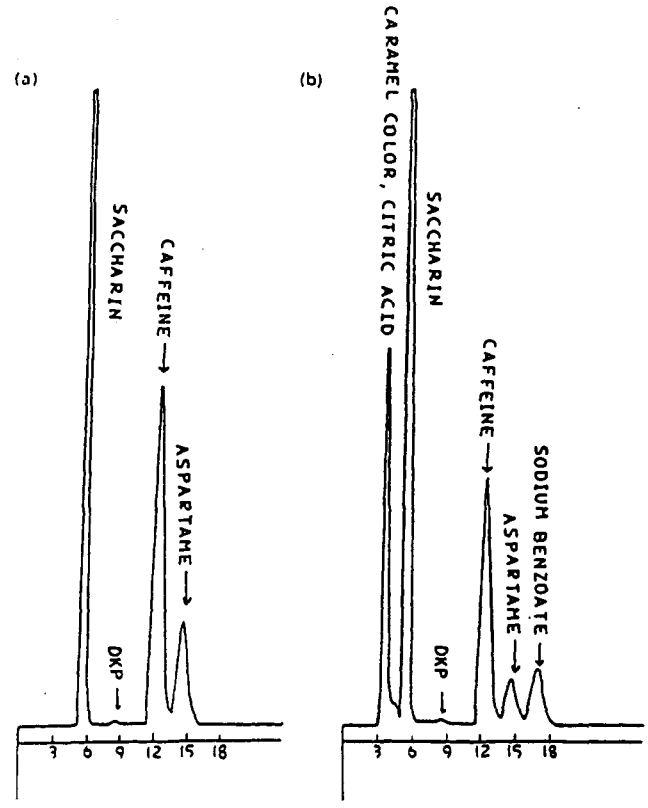

Figure 1. Isocratic analysis of (a) a mixture of standards and (b) the diet cola X. Column: μBondapak C18 (3.9 mm × 30 cm); mobile phase: 0.0125 M KH_2PO_4, pH 3.5/CH_3CN (90:10); flow rate: 0.8 mL/min; temperature: 22 °C; detection: UV 214 nm.

Peak area measurements were used to quantify APM and its degradation products, and least squares linear regression equations were established for each compound on a series of four levels of standard. Calculation of sample concentrations was achieved from the slope and intercept obtained.

RESULTS AND DISCUSSION

Figure 1 part a shows a typical chromatogram of a standard mixture of APM, caffeine, and saccharin. An actual chromatogram for a recently purchased sample of diet cola which was sweetened with a blend of APM and saccharin is shown in Figure 1 part b. On the chromatographic system used in the diet cola, aspartame elutes after caffeine and is followed by sodium benzoate (Figure 1 part b). At pH 3.5, aspartame elutes at about 14 min. The first peak at about 3.4 min contains citric acid and caramel color. The large saccharin peak was observed at about 5.5 min.

When aspartame is heated, it cyclizes to 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP) (Mazur, 1983). One molecule of methanol is liberated during the process. Slow formation of DKP can also be observed when aspartame is dissolved in aqueous solution (Figure 2). In Figure 2 part b, the aspartame sample contains about 2% DKP. It is important to prepare fresh aspartame standard on the day of use or take into account the amount of DKP formed. Recovery experiments were conducted by spiking known quantities of aspartame standards into a diet drink sample and then assaying the sample before and after the addition. Aspartame recovery (%) in the beverage was 99.5%. Reproducibility was determined by injecting a standard solution of aspartame six times. The coefficient of variation was less than 0.3%. For detection of aspartame and its degradation product, 214 nm is a better choice of wavelength than 254 nm because higher response is

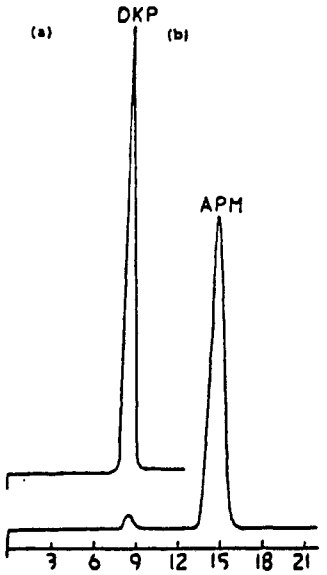

Figure 2. Isocratic analysis of (a) 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP) standard and (b) aspartame (APM) standard which had been standing at 22 °C for 4 h. Chromatographic conditions same as in Figure 1.

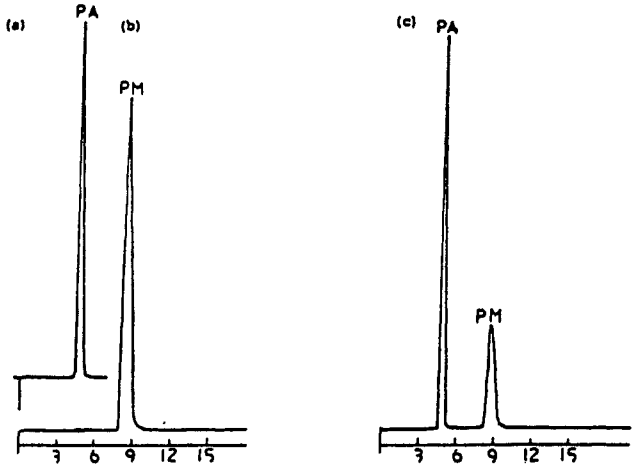

Figure 3. Isocratic analysis of (a) phenylalanine (PA) standard, (b) phenylalanine methyl ester (PM) standard, and (c) phenylalanine methyl ester hydrolyzed at pH 8 for 4 h. Chromatographic conditions same as Figure 1. Peaks: PA = phenylalanine (t_r 4.76 min), PM = phenylalanine methyl ester (t_r 8.43 min).

obtained with the lower wavelength.

The chromatograms in Figure 3 parts a and b are of freshly prepared standards of phenylalanine (PA) and phenylalanine methyl ester (PM), respectively. Figure 3 part c is a chromatogram of the same phenylalanine methyl ester (PM) standard, which was adjusted to pH 8 with diluted NaOH solution and allowed to stand 4 h at 22 °C. The appreciable drop in the peak size of the phenylalanine methyl ester (PM) is obvious when comparing the control and hydrolyzed solutions (Figure 3 parts b and c) and amounts to 70% hydrolysis. The new product obtained from the base-catalyzed hydrolysis corresponds to phenylalanine (PA) when compared to an authentic sample.

When a 0.05% solution of aspartame was adjusted to pH 8 with diluted sodium hydroxide solution at room temperature (22 °C), after 4 h two products were generated (Peaks AP and DKP) at the expense of aspartame (Figure 4 part b). After 24 h there was no aspartame left (Figure 4 part c). Peak DKP, which amounts to 37% hydrolysis of the decomposed aspartame, corresponds to 5-benzyl-3,6-dioxo-2-piperazineacetic acid standard in Figure 4 part a. Peak AP, which was the major product, was presumed

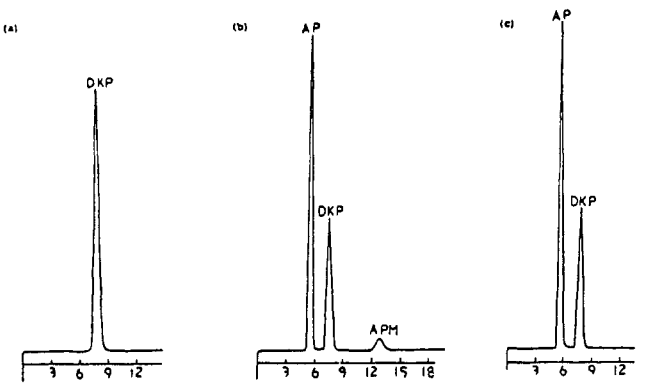

Figure 4. Isocratic analysis of (a) DKP standard, (b) aspartame (APM) hydrolyzed at pH 8 for 4 h, and (c) aspartame hydrolyzed at pH 8 for 24 h. Chromatographic conditions same as in Figure 1. Peaks: DKP = 5-benzyl-3,6-dioxo-2-piperazineacetic acid (t_r 7.56 min), AP = aspartylphenylalanine (t_r 5.52 min), APM = aspartame (t_r 12.59 min).

Table I. Aspartame Levels in Soft Drinks Stored over Months on the Basis of Percentage of the Original Level as 100% Aspartame

	lime–lemon no. 1	lime–lemon no. 2	diet cola no. 1	diet cola no. 2
freshly prepared	100%	100%	100%	100%
1 month	89.7%	87.3%	94%	84.5%
6 months	56.9%	36.9%	38.2%	28.2%
36 months	13.72%	6.40%	4.10%	3.58%

to be the base-catalyzed hydrolysis product, aspartylphenylalanine. The amount of AP was calibrated without isolating a pure sample because AP and DKP were the only products formed from aspartame at pH 8. In contrast, Scherz, and co-workers (1983) reported that equimolar ratios of AP and DKP were formed slowly at room temperature from aspartame in acidic solution.

The identification of the aspartame (APM) and its decomposition products was based on HPLC retention times. Regression equation and correlation coefficient were determined for each standard. Correlation coefficients for all standards were nearly identical with values ranging from 0.9996 to 0.9999. These equations and coefficients were true in the concentration range 0.05–0.3 μg for phenylalanine, 5-benzyl-3,6-dioxo-2-piperazineacetic acid, and aspartylphenylalanine, 0.015–0.090 μg for L-phenylalanine methyl ester, and 0.067–0.4 μg for aspartame.

Of the four brands of soft drink manufactured and purchased in Canada, all have been kept at 22 ± 1 °C for periods of 1 month, 6 months, and 36 months (approximate time after purchase), respectively. The results of the analyses of aspartame in carbonated diet drinks are shown in Table I.

Aspartame appears to have been quite unstable in the beverages for any extended period of time at 22 ± 1 °C. All samples except one contained less than 40% of the label claim for aspartame content in 6 months. Three of the four samples tested contained less than 10% of the original level of aspartame in 36 months. In only 4 weeks, over 10% of the aspartame had decomposed in most samples. However, if the average time between manufacture of APM-sweetened products and the recycling of returnable containers is 4–5 weeks, then most APM-sweetened beverages may be consumed before loss of sweetener becomes a serious problem in soft drink quality.

Figure 5 part a illustrates a chromatogram of a diet cola (no. 1) which had been stored at 22 ± 1 °C for 6 months. Figure 5 part b is the chromatogram of the same brand of soft drink which had been allowed to stand for 36

Table II. Levels of Aspartame and Its Degradation Products in Soft Drinks after Storage

	6 months storage				36 months storage			
	lime–lemon no. 1	lime–lemon no. 2	diet cola no. 1	diet cola no. 2	lime–lemon no.1	lime–lemon no. 2	diet cola no. 1	diet cola no. 2
aspartame (APM)								
label claim, μg/mL	300	490	450	550	300	490	450	550
found, μg/mL	170.66	180.55	171.67	155.34	41.15	31.37	18.44	19.70
% of APM claim	56.89	36.85	38.15	28.24	13.72	6.40	4.10	3.58
L-phenylalanine methyl ester								
found, μg/mL	11.05	21.53	21.26	28.62	5.69	8.59	9.50	13.01
APM equiv	18.14	35.35	34.91	46.99	9.34	14.10	15.60	21.36
% of APM claim	6.05	7.21	7.76	8.54	3.11	2.88	3.47	3.88
DKP								
found, μg/mL	45.76	99.60	96.14	135.66	84.79	145.90	127.50	173.28
APM equiv	51.39	111.85	107.97	152.35	95.22	163.85	143.18	194.59
% of APM claim	17.13	22.83	23.99	27.70	31.74	33.44	31.82	35.38
L-aspartylphenylalanine								
found, μg/mL	52.85	102.75	111.61	158.31	93.91	149.06	141.96	189.05
APM equiv	55.49	107.89	117.19	166.23	98.61	156.51	149.06	198.50
% of APM claim	18.50	22.02	26.04	30.22	32.87	31.94	33.12	36.09
L-phenylalanine								
found, μg/mL	6.75	19.70	29.01	42.22	30.55	67.20	74.00	101.27
APM equiv	12.03	35.11	51.70	75.24	54.44	119.75	131.87	180.46
% of APM claim	4.01	7.16	11.49	13.68	18.15	24.44	29.30	32.81
total aspartame accounted for, %	102.58	96.07	107.43	108.38	99.59	99.10	101.81	111.74

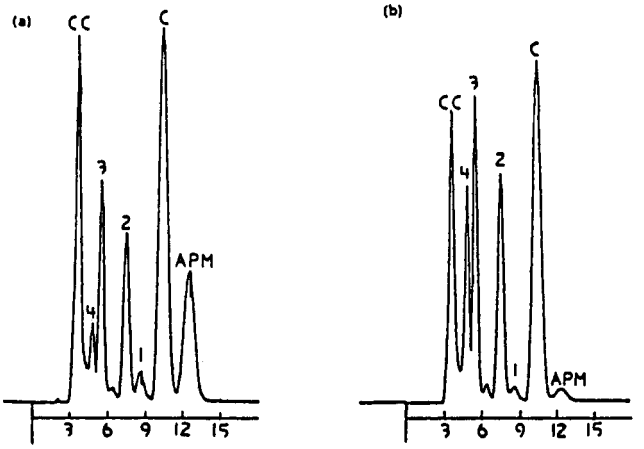

Figure 5. Comparison of HPLC–UV chromatograms of a diet cola (no. 1) which had been stored at 22 °C for (a) 6 months and (b) 36 months. Chromatographic conditions as described in Figure 1. Peaks: APM = aspartame, c = caffeine, CC = caramel color (+ aspartic acid), 1 = phenylalanine methyl ester, 2 = DKP, 3 = aspartylphenylalanine, 4 = phenylalanine.

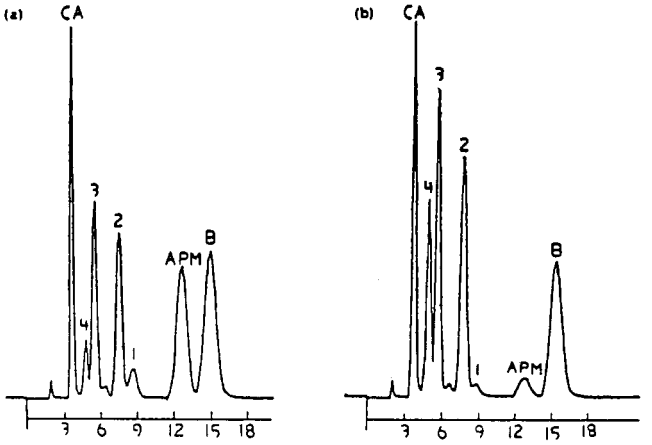

Figure 6. Comparison of HPLC–UV chromatograms of a diet lime–lemon soda (no. 2) which had been stored for (a) 6 months and (b) 36 months. Chromatographic conditions as described in Figure 1. Peaks: APM = aspartame, B = sodium benzoate, CA = citric acid (+ aspartic acid), 1 = phenylalanine methyl ester, 2 = DKP, 3 = aspartylphenylalanine, 4 = phenylalanine.

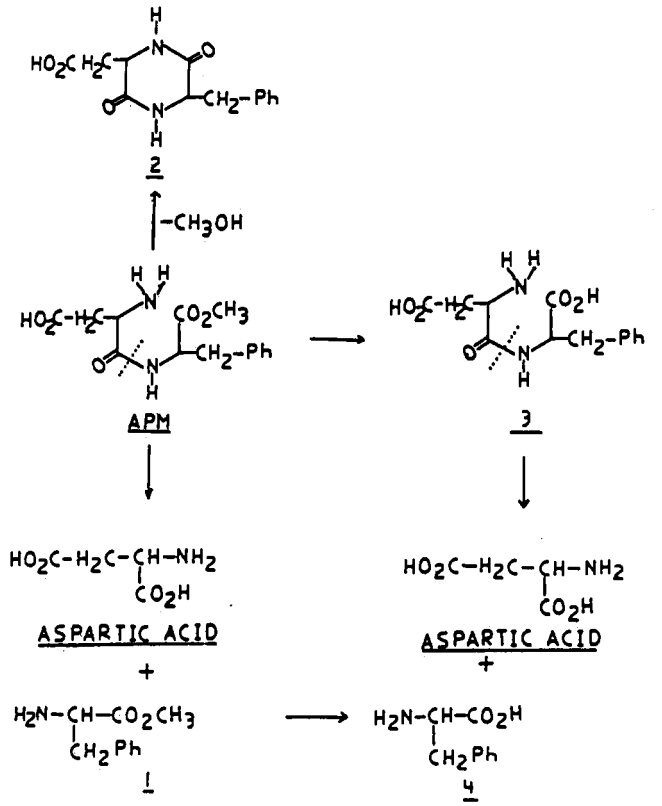

Figure 7. Aspartame deterioration.

months. Similarly, Figure 6 compares the chromatographic pattern obtained for a lime–lemon (no. 2) soda stored 6 and 36 months, respectively. Four breakdown products are identified and their relative proportions determined (Table II). No aspartame condensation product has been identified in this study.

The results of the analyses in Table II indicate that the degradation patterns of aspartame in various types of carbonated beverages appear to be very similar. In most cases, the amounts of unchanged aspartame and the degradation products accounted for almost 100% of the claimed aspartame. This study allowed us to trace the development of various reaction products over a period of time. Compounds **2** and **3** (Figure 7), which correspond

to DKP and aspartylphenylalanine, respectively, are the major products formed in the sample after extended storage periods. L-Phenylalanine methyl ester (peak 1) was a minor product. A significant increase in the level of phenylalanine (peak 4), however, was observed between a period of 6–36 months. The term "APM equiv" in Table II stands for the aspartame equivalent of the amount of breakdown product on a molar basis. It is interesting to note that the same proportion of DKP (37% of the decomposed APM) was obtained at pH 8 and at the low pH of the soft drinks.

The pathways of decomposition of aspartame in the diet soft drinks are depicted in Figure 7. One of the major degradation product is 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP) (2), obtained from cyclization of aspartame, with a resultant loss of methanol. The other degradative pathways involve the formation of phenylalanine methyl ester (1) and aspartylphenylalanine (3), which separately undergo further hydrolysis to give phenylalanine (4).

Registry No. PM, 2577-90-4; DKP, 55102-13-1; AP, 13433-09-5; PA, 63-91-2; aspartame, 22839-47-0.

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Polymerization of Deamidated Peptide Fragments Obtained with the Mild Acid Hydrolysis of Ovalbumin

Naotoshi Matsudomi, Tetsuyuki Sasaki, Atsushi Tanaka, Kunihiko Kobayashi, and Akio Kato*

The mild acid treatment of ovalbumin brought about the deamidation of asparagin and glutamine and the hydrolysis of peptide bonds on either side of aspartic acid. The ovalbumin peptide fragments thus obtained were polymerized by being allowed to stand at room temperature in contact with air. It was suggested that the polymerization of ovalbumin peptide fragments was due to the formation of hydrophobic and disulfide bonds. The polymerized products indicated good functional properties, such as solubility, emulsifying, and foaming properties, because of the higher electrostatic repulsion as a result of deamidation, the better amphiphilic nature due to the proper hydrophilic–hydrophobic arrangement, and the polymerization through hydrophobic and disulfide bonds of ovalbumin peptide fragments.

INTRODUCTION

Some investigators have reported that mild acid treatment of food proteins was very effective to improve their functional properties (Finley, 1975; Wu et al., 1976; Matsudomi et al., 1982). Finley (1975) suggested a mild acid treatment of wheat gluten to increase its solubility in fruit-based acidic beverages. Wu et al. (1976) found a significant improvement in the emulsifying and foaming properties of wheat gluten by mild acid hydrolysis. It is well-known that mild acid treatment of proteins results in the deamidation of glutamine and asparagine residues and a concomitant cleavage of peptide bonds. On either side of aspartic acid, peptide bonds may be cleaved at a rate at least 100 times greater than other peptide bonds under carefully controlled conditions in diluted acid (Schultz et al., 1962; Han et al., 1983). Therefore, this treatment appears to be more suitable for controlled hydrolysis of proteins than protease. A mild acid hydrolysis may bring about (1) increased solubility of proteins mainly due to the higher electrostatic repulsion as a result of deamidation of glutamine and asparagine, and (2) the higher amphiphilic nature due to the exposed hydrophobic amino acid residues as a result of either denaturation or cleavage of peptide bonds.

In addition to these advantages, we found some sort of polymerization of peptide fragments in the mild acid hydrolysates of ovalbumin. This reaction can be utilized as an useful tool to fabricate new functional proteins. The present paper describes the mechanism of polymerization and the properties of resultant peptide products.

MATERIALS AND METHODS

Ovalbumin was prepared from egg white by a crystallization method in sodium sulfate and recrystallized five times (Kekwick and Cannan, 1936).

The mild acid hydrolysis of ovalbumin was carried out as follows. Ovalbumin was dissolved in a concentration of 1 mg per mL of 0.03 N HCl. Ovalbumin solution was sealed in 10-mL amples in vacuo and placed in an oven at 115 °C. At various time intervals, ampules were removed and cooled.

Ovalbumin solution (3 mL) was immediately applied to a Sephadex G-100 column (0.8 × 112 cm) equilibrated with 0.03 N HCl. Another 3 mL of ovalbumin solution was allowed to stand at room temperature in contact with air for a given time before application to a Sephadex G-100

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