Food Chemistry **24** (1987) 187–196

Studies on the Maillard Browning Reaction Between Aspartame and Glucose*

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(Received 9 July 1986; accepted after revision 15 September 1986)

ABSTRACT

HPLC and column chromatography were used to study the Maillard browning reaction between aspartame and glucose. The aspartame concentrations remaining in the reaction mixture after heating were determined by the HPLC method developed in this experiment. The activation energy of this reaction was thus estimated to be 18·89 ± 0·96 kcal mol^{-1}. Four Schiff bases were isolated and purified by the successive application of Sep-Pak C-18 cartridge and Sephadex G-25 column chromatography. By FAB–MS, these four Schiff bases were interpreted as N-(1-deoxy-D-fructo-1-yl)-aspartame, N-(1-deoxy-D-fructo-1-yl)-phenylalanine methyl ester, N-(1-deoxy-D-fructo-1-yl)-aspartic acid and N-(1-deoxy-D-fructo-1-yl)-phenylalanine, respectively.

INTRODUCTION

Aspartame (*N*-L-α-aspartyl-1-L-phenylalanine methyl ester), a high potency non-carbohydrate sweetener, has gained approval for use as a sweetener in foods. Aspartame is currently used widely in low-calorie foods (Hussein *et al.*, 1984).

The stability of aspartame in liquid systems has been reported as a function of time, temperature, pH and available moisture (Homler, 1984).

* New Jersey Agricultural Experiment Station Publication No. D-10205-7-86.

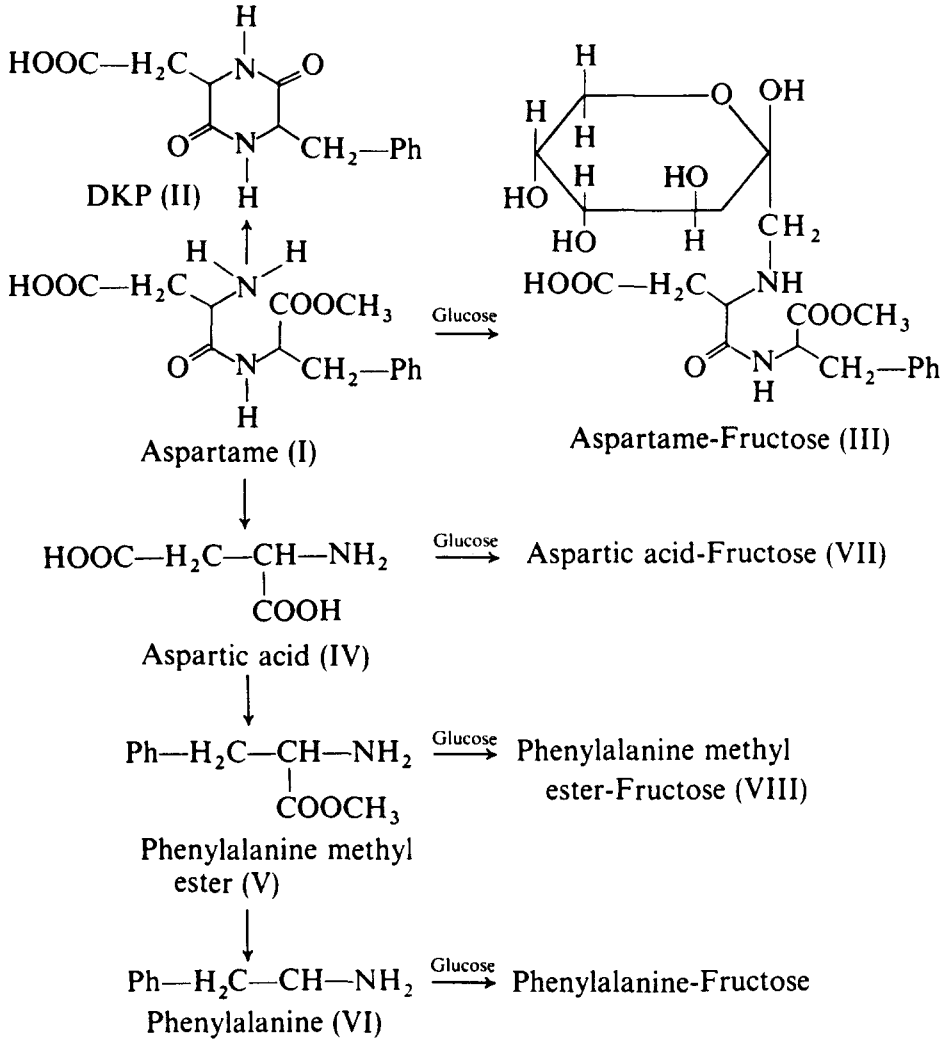

Fig. 1. Mechanism of aspartame degradation and formation of Schiff bases.

The degradation mechanism of aspartame (I) in solution was proposed by Homler (1984). The major thermal degradation products were identified as aspartylphenylalanine, DKP (5-benzyl-3,6-dioxo-2-piperazineacetic acid) (II), aspartic acid (IV), phenylalanine methyl ester (V) and phenylalanine (VI) (Fig. 1).

Aspartame also interacts with food components which results in the loss of sweetening power, off-flavors, or undesirable color change. Hussein *et al.* (1984) reported that flavor aldehydes react with aspartame in chewing gums. Stamp & Labuza (1983) reported that glucose in aqueous solution reacts with aspartame. However, the reaction products were not identified.

Fox *et al.* (1976) published the first application of high performance liquid chromatography (HPLC) with a strong cation exchange column to analyze aspartame. Scherz *et al.* (1983) also developed two methods, based on liquid chromatography, for the analysis of the peptide sweetener aspartame. Webb & Beckman (1984) reported the separation and quantitation of aspartame in beverage and beverage mixes by reversed phase liquid chromatography. Recently, Tsang *et al.* (1985) presented a rapid and simple isocratic solvent system for the determination of aspartame and its breakdown products in carbonated and soft drinks.

The objective of this study is to apply the chromatographic techniques, involving reversed phase high performance liquid chromatography and column chromatography, to study the Maillard reaction between aspartame and glucose from the viewpoint of kinetics and chemical structure.

MATERIALS AND METHODS

Sample preparation

The model system in this study was a 20% methanol in water solution of equal molar (0·1 M) glucose (Aldrich Chemical Co, Milwaukee, WI, USA) and aspartame (L-aspartyl-L-phenylalanine methyl ester, G. D. Searle Co. Chicago, IL USA). Methanol was used to increase the solubility of aspartame. The sample solution was subjected to heat at three different temperatures (80°C, 90°C and 100°C) for 10 h. One-millilitre aliquots of reacted solution were taken at 2-hourly intervals.

A Sep-Pak C-18 cartridge (Waters Associates, Milford, MA, USA) was used for the separation of colorless aspartame and its degradation products from the brown-colored reaction products. The clear filtrate from the Sep-Pak was directly subjected to HPLC analysis. The brown colored material was absorbed on the Sep-Pak, was washed with water and 10% methanol successively, and finally eluted with 100% methanol. The colored material was further analyzed by Sephadex G-25 column chromatography.

Phenylalanine, phenylalanine methyl · ester and aspartic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DKP was prepared as per the method performed by Tsang *et al.* (1985). Reference Schiff bases, ASP–FRU, PHE–FRU and PM–FRU, were prepared by mixing glucose with aspartic acid, phenylalanine and phenylalanine methyl ester, respectively, with adjustment of the Aw to 0·68 by storing in a sealed Mason jar which contained saturated NaCl solution at 25°C. The prepared

mixtures were heated in an oven at 100°C for 2 h. The Schiff bases were purified by Sep-Pak C-18 cartridge, as was described for ASP–FRU.

Column chromatography

Column chromatography was performed on a Sephadex G-25 (Pharmacia Chemical Co., Piscataway, USA) column. Sephadex G-25 gel was equilibrated in 10% methanol in water overnight to get a fixed pore size. The column was eluted with 10% methanol in water at the rate of 1 ml min^{-1}.

HPLC

HPLC analysis was carried out with a Waters Associates liquid chromatograph equipped with two M600 pumps, an M660 solvent programmer and a Varian 2050 variable wavelength detector (254 nm). A Whatman Partisil ODS-3 reversed phase column (4·6 mm inside diameter × 30 cm) of 5 μm average particle size was used. A linear gradient programme from 100% acetonitrile : 0·02N acetic acid (1:9) to 50% methanol: acetonitrile: 0·02N acetic acid (9:9:2) in 10 min was chosen in conjunction with a flow rate of 1 ml min^{-1}.

Fast atom bombardment–mass spectrometry (FAB–MS)

FAB–MS spectra were obtained on a VG 7070 Mass Spectrometer equipped with an Ion Tech Saddle Field FAB gun. The sample was dissolved in 'magic bullet' (1:3 mixture of dithioerythritol and dithiothreitol) matrix, and applied on a copper probe and bombarded with 8 kV Xenon atoms.

^{13}C-NMR

^{13}C-NMR was recorded at 100 MHz with a Varian 400 Inpulsed FT–NMR spectrometer. Dimethyl sulfoxide-d_6 was used as the solvent and tetramethylsilane served as an internal reference.

RESULTS AND DISCUSSION

Chromatographic methods, reversed phase liquid chromatography and Sephadex G-25 column chromatography were used to study the reaction between aspartame and glucose.

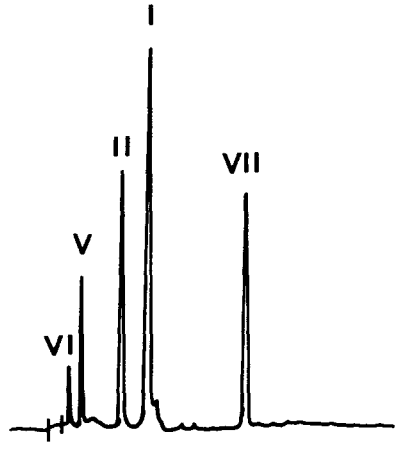

Fig. 2. HPLC profile of aspartame and its degradation products. Heating condition: 80°C, 8 h.

As reported by Arabshahi & Lund (1985), two common mistreatments of kinetic data are: (1) using per cent retention instead of raw data in determining rate constants, (2) ignoring the variability in rate constants in calculating activation energy. To avoid the first mistreatment, a simple reversed phase HPLC method was developed to determine the exact concentration of aspartame that remained in the reaction mixture. A gradient mobile phase from 10% acetonitrile in 0·02N HCl to 45% acetonitrile and 45% methanol in 0·02N HCl in 15 min was used.

As shown in Fig. 2, aspartame was well separated from all the other degradation products. Thus the aspartame concentration was accurately determined for the kinetic analysis. In addition to the concentration determination, some interesting phenomena were also observed. In the early stage of heating, DKP and an unknown compound were generated. As the heating treatment progressed, concentration of these two compounds increased significantly, whereas the concentration of aspartame simultaneously decreased.

The results of this experiment showed the polarity of the unidentified compound to be less than that of aspartame.

The unknown peak was collected by a semi-preparative Whatman ODS-3 column using the same HPLC conditions as described previously. The collected clear solution was lyophylized and analyzed by FAB–MS. The molecular weight of this unidentified compound was determined to be 431 by FAB–MS: m/z 432 for $[MH]^+$, m/z 454 for $[MNa]^+$ and m/z 470 for $[MK]^+$ as listed in Table 1. The chemical structure of this compound remains to be elucidated.

For calculating the Arrehnius activation energy (E_a), the two-step regression method was used. This method starts from regressing aspartame

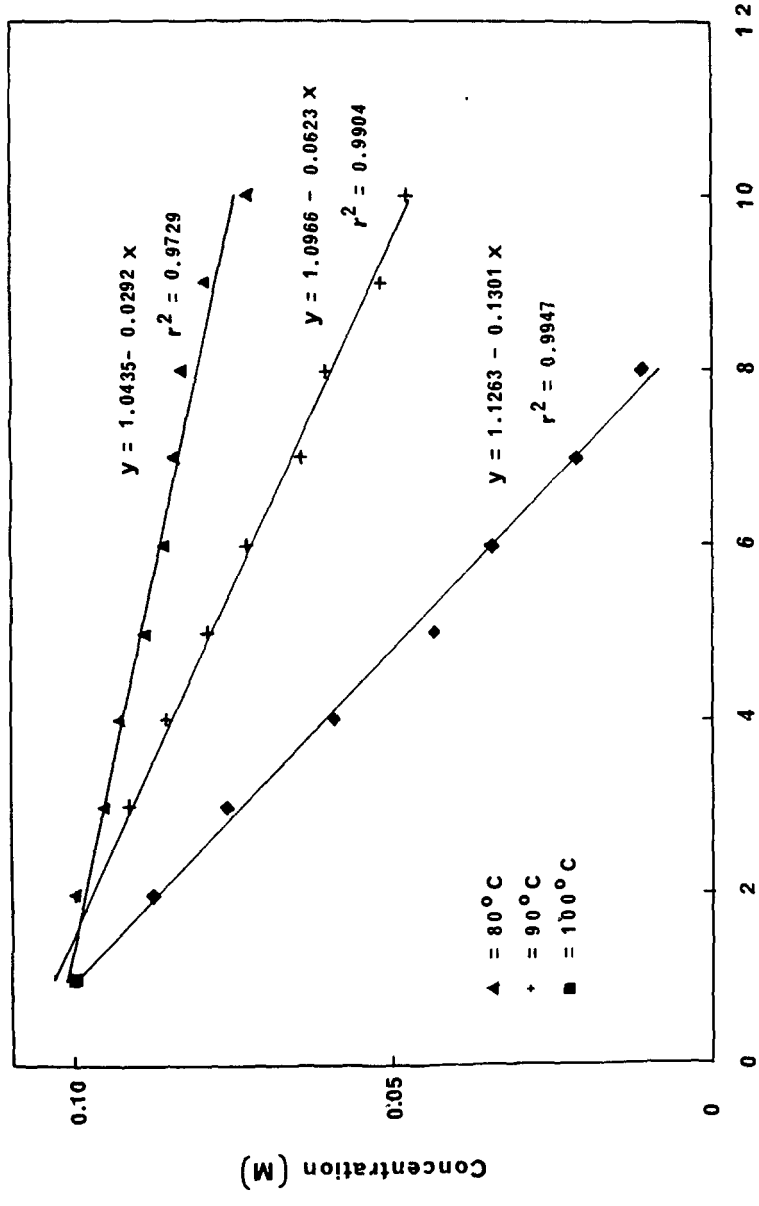

Fig. 3. The decrease of aspartame concentration in aqueous model.

TABLE 1
FAB–MS of Unidentified Compound

m/z	*Rel. int.*	*Interpretation*
432	100	$[M+H]^+$
454	35	$[M+Na]^+$
470	70	$[M+K]^+$

concentration on time at constant temperature to determine the rate constant, K, and then regressing $\log K$ on reciprocal temperature to determine E_a. As shown in Fig. 3, the reaction rates at 80°C, 90°C and 100°C were estimated to be $0{\cdot}0292 \pm 0{\cdot}0011$, $0{\cdot}0623 \pm 0{\cdot}0014$ and $0{\cdot}1306 \pm 0{\cdot}0034$ M h^{-1}, respectively. The small 95% confidence intervals and small standard deviation for all K values indicate little analytical error. The activation energy thus calculated was $18{\cdot}89 \pm 0{\cdot}96$ kcal mol^{-1}. This value is larger than the reported activation energy, 15 kcal mol^{-1}, for most hydrolysis, but smaller than that of non-enzymic browning, 25–50 kcal mol^{-1} (Saguy & Karel, 1983). These data indicate that both hydrolysis and non-enzymatic browning reactions may occur simultaneously during heating.

From the visual observation, brown color starts to develop after 7 hours' heating at 80°C, 4 hours' at 90°C and 2 hours' at 100°C. The brown-colored compound was collected and purified using Sep-Pak C-18 cartridge as described previously, and then subjected to the Sephadex G-25 column chromatographic separation. Only one band was eluted for the sample collected from the early stage of the reaction mixture. However, two brown-colored bands were eluted from the sample collected at the later stage of heating. The chemical structures of both bands were studied by the application of FAB–MS and ^{13}C-NMR techniques.

The FAB–MS data for the first band are listed in Table 2. The molecular weight of this compound was found to be 456, m/z 457 for $[MH]^+$. The

TABLE 2
FAB–MS of Aspartame-Fructose

m/z	*Rel. int.*	*Interpretation*
495	100	$[M+K]^+$
479	30	$[M+Na]^+$
457	80	$[M+H]^+$
439	40	$[M+H-H_2O]^+$
421	60	$[M+H-2H_2O]$

TABLE 3
^{13}C-NMR Chemical Shifts for the Sugar Moieties in APM–FRU, ASP–FRU and FRU

Carbon	*APM–FRU*	*ASP–FRU*[a]	*β-D-Fructopyranose*[a]
C1	52·37	53·09	65·5
C2	97·09	95·31	99·1
C3	72·14	70·46	69·3
C4	70·82	69·51	71·1
C5	70·59	69·01	70·4
C6	61·51	64·12	64·6

APM, Aspartame. ASP, Aspartic acid. FRU, Fructose.
[a] Röper *et al.* (1983).

molecular ion was confirmed by the presence of m/z 479 for $[MNa]^+$ and m/z 495 for $[MK]^+$. The m/z 439 peak was interpreted as $[MH - H_2O]^+$ and m/z 421 as $[MH - 2H_2O]^+$.

The ^{13}C–NMR data are listed in Table 3. It was found that a group of signals ranging from 70·59 ppm to 72·14 ppm were attributable to C-3,4,5 of β-D-fructo-pyranose. In addition, the presence of 52·37 ppm for C-1, 97·06 ppm for C-2 and 61·51 ppm for C-6 confirms that the glucose, which attaches to aspartame, has been transformed to β-D-fructo-pyranose. Furthermore, the signal at 52·37 ppm strongly indicates that the C-1 carbon is the site where fructose and aspartame combine. It is noticeable that there is no significant difference in the chemical shift between the carbon atoms of authentic aspartame and the aspartame moiety of aspartame-fructose. The data indicate that the structure of the aspartame moiety did not change during the Maillard browning reaction. In addition, the ^{13}C-NMR chemical shift of aspartame-fructose was found to be very similar to that of aspartic acid-fructose reported by Röper *et al.* (1983). Thus, the structure of

TABLE 4
Identification of Aspartame and its Degradation Products by HPLC

Peaks	*Retention time*		*Interpretation*
	Author	*Authentic*	
I	9·42	9·42	Aspartame
II	7·74	7·74	DKP
V	5·04	5·04	PM
VI	4·22	4·22	PHE
VII	15·85	—	Unknown

TABLE 5
FAB–MS of Reference ASP–FRU, PHE–FRU and PM–FRU

	Schiff bases		
	ASP–FRU	*PHE–FRU*	*PM–FRU*
$[M + K]^+$	334	366	380
$[M + Na]^+$	318	350	364
$[M + H]^+$	296	328	342
$[M + H - H_2O]^+$	278	310	324

the brown compound was identified as *N*-(1-deoxy-D-fructo-1-yl)aspartame (III) (Fig. 1).

Three Maillard browning Schiff bases in the second band on the Sephadex G-25 column were identified by FAB–MS. Three free amino group-containing compounds—aspartic acid(IV), phenylalanine methyl ester(V) and phenylalanine(VI)—have been identified by the mass spectrum and phenylalanine and phenylalanine methyl ester were also identified by comparing the HPLC retention time with that of authentic compounds (Table 4). The molecular weights of these three Schiff bases were confirmed and compared to the FAB–MS data (Table 5) of standards which were prepared in this laboratory.

According to the interpretation of aspartame-fructose, it is reasonable to predict that Maillard browning reaction occurs in the later stage of this reaction. Thus, *m/z* 296 $[MH]^+$ was interpreted as *N*-(1-deoxy-D-fructo-1-yl)-aspartic acid(VII), *m/z* 342 $[MH]^+$ as *N*-(1-deoxy-D-fructo-1-yl)-phenylalanine methyl ester(VIII) and *m/z* 328 $[MH]^+$ as *N*-(1-deoxy-D-fructo-1-yl)-phenylalanine(IV) as shown in Table 6.

As a conclusion, in the early stage of the reaction, aspartame reacted with glucose to form a Schiff base. In the later stage, following the thermal

TABLE 6
FAB–MS of Schiff Base from Aspartic Acid, Phenylalanine and Phenylalanine Methyl Ester

m/z	*Rel. int.*	*Interpretation*	*Structure*
296	52	$[M + H]^+$	ASP–FRU
328	100	$[M + H]^+$	PHE–FRU
342	30	$[M + H]^+$	PM–FRU

ASP, Aspartic acid.
PHE, Phenylalanine.
PM, Phenylalanine methyl ester.
FRU, Fructose.

degradation reaction, the breakdown products were produced. These breakdown products then react with glucose and generate three corresponding Schiff bases.

ACKNOWLEDGEMENT

We thank New Jersey Agricultural Experiment Station for financial support.

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