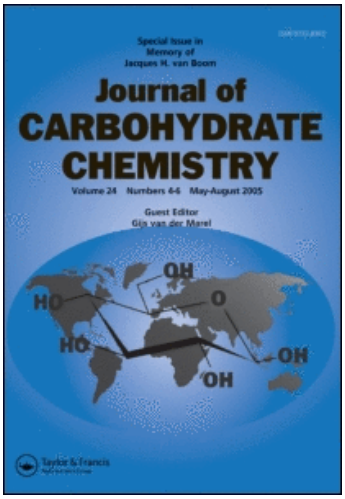

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Maillard Reaction: Investigation of the Chemical Structure of Melanoioins Synthesized from D-Xylose and Glycine Using ^{13}C and ^{15}N Specifically Labeled Reactants

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MAILLARD REACTION: INVESTIGATION OF THE CHEMICAL STRUCTURE OF MELANOIDINS SYNTHESIZED FROM D-XYLOSE AND GLYCINE USING ^{13}C AND ^{15}N SPECIFICALLY LABELED REACTANTS[1]

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ABSTRACT

Melanoidins were isolated in 36% yield w/w from molar solution of D-xylose and glycine-2-^{13}C (A); D-xylose and glycine-1-^{13}C (B); D-xylose-1-^{13}C and glycine (C); D-xylose and glycine (D); D-xylose and glycine-^{15}N (E). Each solution was kept at 68°C until complete disappearance of xylose as evidenced by NMR. ^{13}C and ^{15}N solid state nuclear magnetic resonance and diffuse reflectance infrared spectrometry were used in their structural elucidation before and after basic and acid hydrolysis. Both C-1 and C-2 of glycine were incorporated into the polymers. In the ^{13}C CP-MAS NMR spectra, C-1 gave a single peak in the polymer at 171.3 ppm, while C-2 gave three at 48.1, 31.2 and 22.5 ppm. Area measurements of the respective peaks indicated that 50% of the incorporated glycine had undergone decarboxylation. C-1 of xylose was incorporated into the polymers mainly as two types of carbons at 68.8 ppm (CHOH, C-OH) and at 133.3 ppm (unsaturated C). Hydrolysis (6N HCl) led to a 20% reduction in weight of the melanoidins, a decrease of 2% in C and 10% in N. ^{13}C CP-MAS NMR revealed after hydrolysis of D, the disappearance of signals at 69, 110, 152, 172 and 200 ppm. Hydrolysis of A and B reduced all signals originating from C-1 and C-2 of glycine, while hydrolysis of C reduced only the signal of 68.8 ppm. ^{15}N CP-MAS NMR of hydrolyzed E showed a greatly reduced amide resonance at 100 ppm, with more pyrrole or imino N. DR-IR showed a reduction in both the 1625 and 1550 cm^{-1} bands with a concurrent appearance of a 1715 cm^{-1} band.

INTRODUCTION

Slow progress has been made in the elucidation of the chemical nature of melanoidins. The structure of these high molecular weight polymers formed in the Maillard reaction is dependent on factors such as time, temperature, concentration, pH and nature of reactants. As a result of this complexity, few structures have been proposed. The earlier suggestions that melanoidins contained a purine core,[2] or furan ring repeating units[3] have now been abandoned in favor of a more aliphatic structure[4] with enediols and eneamines postulated as the main unsaturated features.[5] Strong caution, however, has to be exercised when comparing structures of melanoidins synthesized under different reaction conditions. Recent studies[6,7] have shown a definite increase in unsaturation with an increase in time and temperature of reaction.

The purpose of this study is to investigate, using ^{13}C and ^{15}N specifically labeled substrates, the chemical structure of melanoidins obtained by reaction of molar solutions of D-xylose and glycine at 68 °C for six weeks, corresponding to the complete disappearance of xylose. The following system were used: D-xylose and glycine-2-^{13}C (A); D-xylose and glycine-1-^{13}C (B); D-xylose-1-^{13}C and glycine (C); D-xylose and glycine (D); D-xylose and glycine-^{15}N (E).

RESULTS AND DISCUSSION

Labeling Results

Preliminary results[8] using a 30% enrichment of 1-^{13}C and 2-^{13}C glycine in the reaction with xylose, had shown after a reaction time of ten days, that C-1 of glycine only contributed to one signal (172 ppm) in the ^{13}C CP-MAS NMR spectrum of the melanoidin, while C-2 of glycine contributed to several signals in the 0-60 ppm region. When 1-^{13}C-glucose (30% enrichment), was reacted with glycine, the ^{13}C CP-MAS spectrum of the resulting melanoidin showed peaks at 200, 173, 150, 132, 110, 68 and 13 ppm.

More recently, under different reaction conditions, it was reported[4,9] based on solution ^{13}C NMR studies, that both carbon atoms of glycine were incorporated into the polymers obtained from glucose and glycine and that C-1 of D-glucose appeared as a methyl group, probably arising from a 2,3 enolisation of the Amadori compound. In the chemistry of caramel preparation, it was found that C-1 of the sugar is scrambled during melanoidin formation.[10]

The present study is a continuation of our preliminary work, on the use of ^{13}C and ^{15}N specifically labeled reactants in the structural elucidation of insoluble melanoidins formed in a reaction of equimolar amounts of xylose and glycine kept at 68°C.

The insoluble character of the melanoidins precluded the use of purification systems such as gel filtration or electrophoresis. Filtration, followed by thorough washing with water was the only purification step. Melanoidins were obtained in 36% yield (weight/total weight of starting materials). Dialysis of the filtrate showed the presence of polymeric material amounting to no more than 2%. The total low molecular weight water soluble material consisted of unreacted glycine and a twelve carbon eneaminol (Fig. 1D).[11]

As previously reported in the reaction of glycine-1-^{13}C with xylose,[8] when a reaction time of 10 days instead of 42 days was used, the C-1 of glycine contributed only to the 172 ppm signal in the ^{13}C CP-MAS NMR spectrum of the polymer B (Fig. 2, B2). The latter spectrum was obtained by subtraction (see experimental) of the spectrum of the unlabeled polymer (Fig. 2, D) from the labeled one (Fig. 2 B1). As the labeled melanoidins were synthesized using 30% enrichment, this subtraction eliminates the contributions of the natural abundance spectrum from the spectrum of the labeled melanoidin.[12] Feather and Nelson[9] had likewise observed only one signal in the carboxyl C region in the solution spectrum obtained from the non-dialyzable polymer from D-glucose and glycine-1-^{13}C. The ^{13}C solution spectrum of the total water soluble material (Fig. 1B), showed as expected the signals at 173.2 ppm for COOH of glycine as well as a signal at 169.69 ppm corresponding to C-7 in the eneaminol,[11] in addition to a small signal corresponding to C-2 of glycine (natural abundance). The presence of the latter signal is due to the large

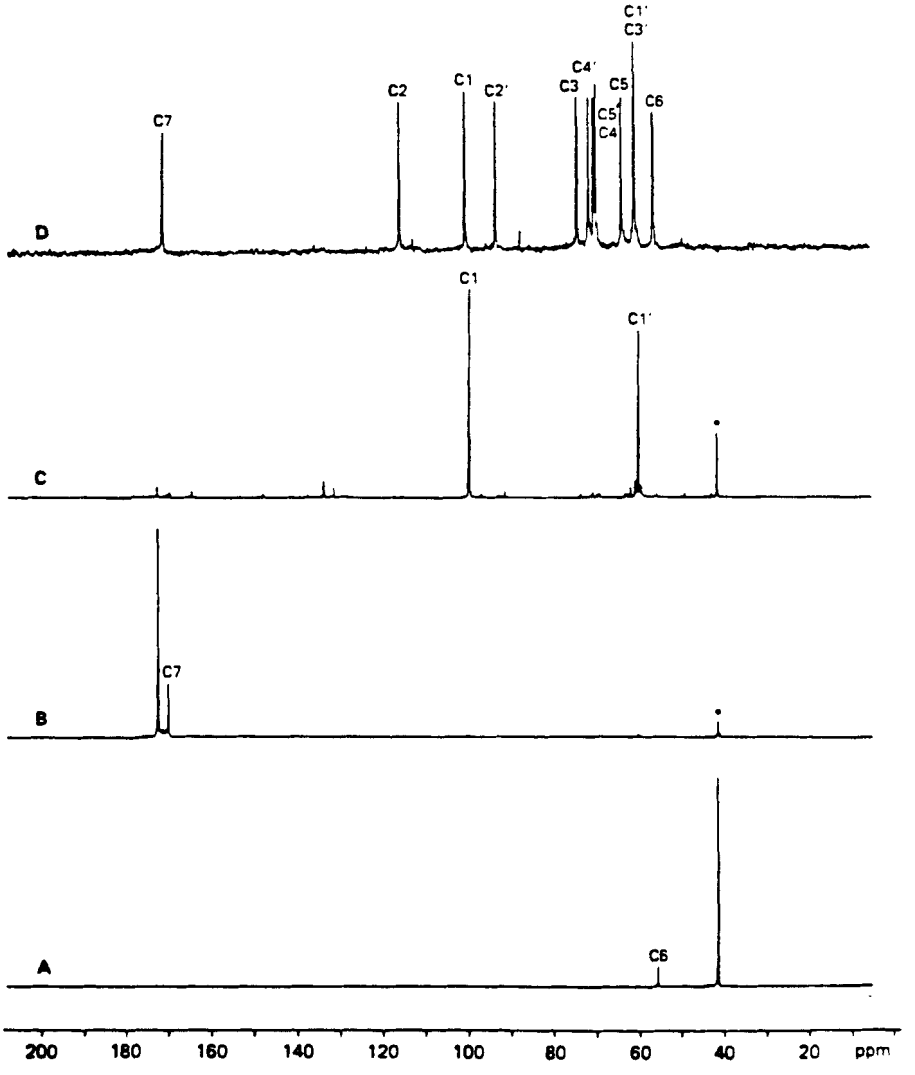

Fig. 1. ^{13}C solution NMR spectra of total water soluble fractions (A, B, C) of melanoidins A, B and C and the 12C eneaminol D.

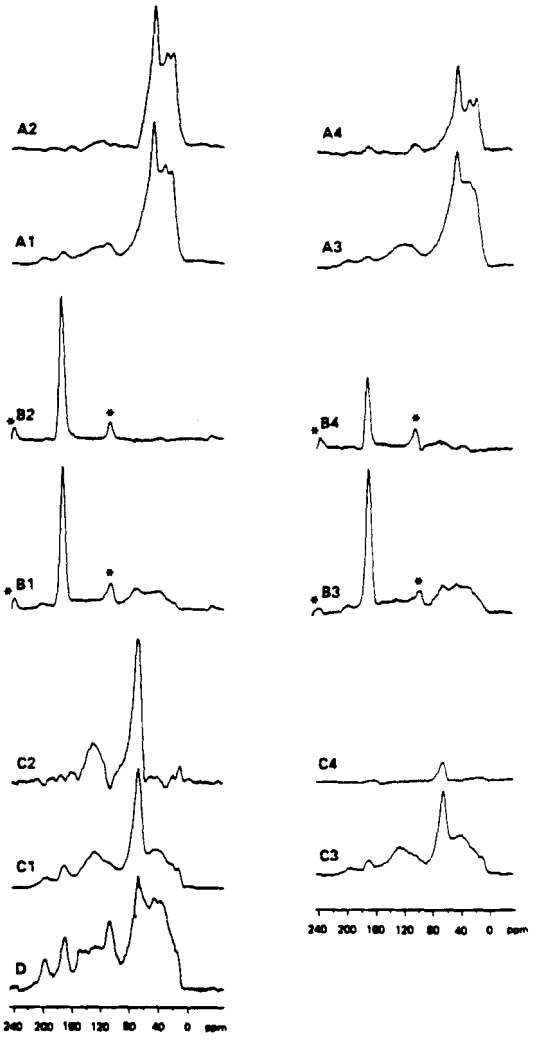

Fig. 2 ^{13}C CP-MAS NMR spectra of melanoidin A (A1); B (B1); C (C1) and D (D). A2, B2 and C2 are ^{13}C spectra obtained by difference: A2=A1-D; B2=B1-D; C2=C1-D. ^{13}C CP-MAS NMR spectra of HCl hydrolyzed melanoidins A (A3); B (B3); C (C3). A4, B4 and C4 are spectra obtained by difference: A4=A1-A3; B4=B1-B3; C4=C1-C3. *Spinning side bands.

amount of unreacted glycine still present after the complete disappearance of xylose.

When glycine-2-^{13}C was reacted with xylose, the C-2 of glycine contributed in melanoidin A primarily to signals in the 0-60 ppm region (Fig. 2, A2) as previously reported,[8] while the spectrum of the soluble material (Fig. 1A), showed in addition to C-2 of unreacted glycine (42.5 ppm), a signal at 55.3 ppm corresponding to C-6 of the eneaminol.[11] A detailed examination of the solid state NMR spectrum of melanoidin A (Fig. 2, A2), reveals the presence of at least three broad peaks, with maxima at 48.1; 31.2; and 22.5 ppm which must correspond to C bearing H, since a spectrum (not shown), obtained using a 40 μsec. delay between contact and acquisition time showed no signals. The 48.1 ppm peak may be attributed to C-2 carbons of an NH substituted glycine moiety. In fructosyl-glycine, the corresponding C occurs at 50 ppm.[13] The peaks at 31 and 22 ppm on the other hand may be attributed to methylene or methyl groups obtained probably as a result of the decarboxylation. Area measurements of the respective peaks 48.1 versus 31.2 and 22.5 ppm would indicate that 50% of the incorporated glycine had lost a COOH group. These results are consistent with those obtained by Feather and Huang[14] and Kato et al.[4] in the reaction of D-glucose with glycine. In a reaction of D-xylose and glycine in the presence of sodium bicarbonate at 100°C for 4 hrs. Kato et al.[5] reported that decarboxylation accounted for half a mole per mole of glycine.

When D-xylose-1-^{13}C was reacted with glycine the resulting ^{13}C CP-MAS spectrum of melanoidin C (Fig. 2, C1) showed several peaks. Fig. 2, C2, however, the difference between spectra D and C1, shows mainly three peaks at 68.8 ppm (major resonance which may be attributed to CHOH or COH); 133.3 ppm (unsaturated C); and 13.8 ppm (very minor peak, probably a CH_3 resonance). Based on results obtained in the gluco series where chemical shifts for C-1 in N-D-glucosyl-glycine and 1-deoxy-1-L-glycine-D-fructose have been reported to be 90.1 and 54.3 ppm, respectively,[13] the three main peaks arising from C-1 rule out an amino xyloside or an Amadori compound as principal unit in the melanoidin from D-xylose and glycine. Non-dialyzable melanoidins prepared from glycine-glucose- 1-^{13}C, showed broad signals centered at 13, 21, 71, 132, 174 and 197 ppm.[4] Others have reported that C-1

resulted in methyl, methylene, and unsaturated C in the corresponding melanoidin.[10] The ^{13}C solution spectrum of the total water soluble material (Fig. 1 C), showed as expected mainly the resonances for C-1 and C-1' of the eneaminol[11] in addition to C-2 of glycine (natural abundance).

Degradation Results

In order to gain further insight into the chemical nature of these melanoidins, both basic and acid hydrolyses were conducted. Treatment of the unlabeled melanoidin D with 1M NaOH at 50°C for 6 hrs. under N_2 atmosphere, conditions reported to hydrolyze ester linkages, did not cause any visible change in the ^{13}C CP-MAS NMR spectrum run under normal conditions or in the spectrum run using a 40 μsec delay between contact and acquisition. Similarly, the diffuse reflectance (DR) infrared spectra of the original and NaOH treated samples were almost identical as were microanalytical data of the ash free melanoidin before and after treatment. Amino acid analysis of the hydrolysate revealed only traces of glycine and ammonia. These results indicate the absence of ester linkages in melanoidin.

Acid hydrolysis under conditions used to cleave peptide linkages in proteins resulted in a 20% reduction in weight of the melanoidin, with a 2% decrease in C and a 10% in N. Natural abundance ^{13}C NMR revealed after hydrolysis (Fig. 3.2A, B, C) changes in the NMR spectra when compared to the corresponding spectra Fig. 3.1A, B, C of the original melanoidin run under similar conditions. The changes in the chemical structure of the HCl treated melanoidin may be more readily recognized in spectrum Fig. 3.3 (difference between 2A and 1A), which showed peaks at 200, 172, 152, 110 and 70 ppm. The difference, however, is not the result of a subtraction of absolute intensities; the spectra were scaled such that the difference showed no negative regions. This implies an assumption that only material is lost on hydrolysis with none formed as a result of it, which may be an oversimplification. The DR-IR spectrum of the original melanoidin showed relevant bands at 1700, 1625, and 1550 cm^{-1} (Fig. 4.I). The two broad bands at 1625 and 1550 may be assigned at least partially to the amide I and II bands respectively.[16,17] The presence of bands at

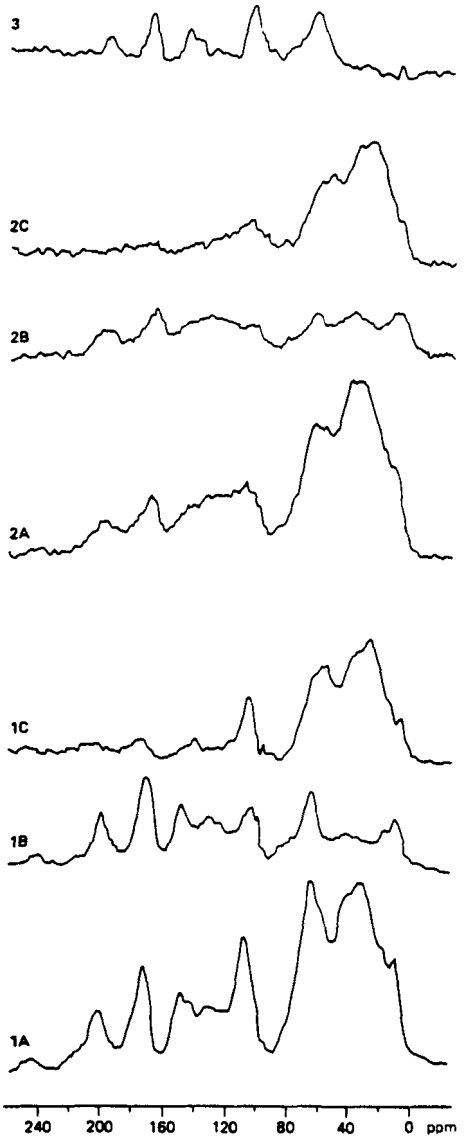

Fig. 3 ^{13}C CP-MAS NMR spectra of melanoidin D; before (1A, 1B, 1C) and after 6N HCl hydrolysis (2A, 2B, 2C). Spectra 1A and 2A were recorded without delay and 1B and 2B with 40 μsec. delay between contact time and acquisition. Spectra C=A-B. Spectra 3=1A-2A.

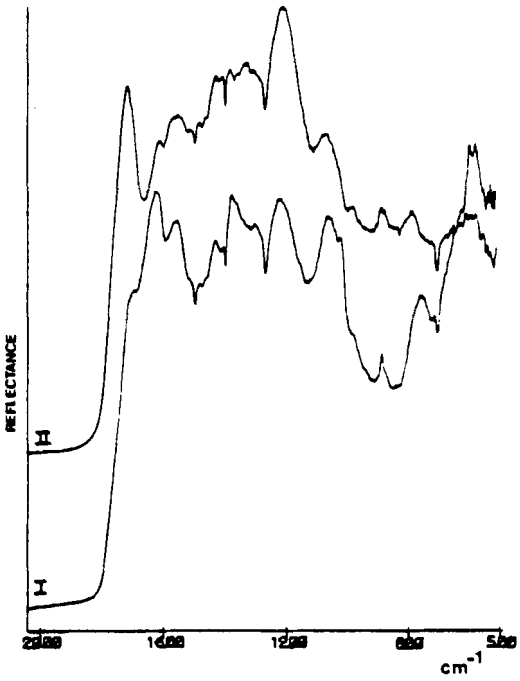

Fig. 4. Partial diffuse reflectance infrared spectra. Melanoidin D (I); HCl hydrolyzed melanoidin D (II).

1220, 745 and 620 cm^{-1}, Amide III, V, IV and VI would corroborate this assignment. The 1700 cm^{-1} band is very likely the keto C=O band. Upon 6N HCl hydrolysis, the 1625 and 1550 bands are greatly reduced, with a concurrent appearance of a strong band at 1715 cm^{-1} (Fig. 4.II). In melanoidins synthesized from glucose and glycine, little or no effects on these two bands was reported.[18] This disparity is very likely a consequence of a different experimental method rather than a result of the use of a different carbohydrate starting material. Similarly, no conclusion on the fate of the two bands at 1625 and 1550 cm^{-1} could be reached from IR of KBr disks. In the ^{13}C CP-MAS NMR spectrum, hydrolysis seems to have removed all CH carbons (acetal or furan C) at 110 ppm (Fig. 3, 2C) in addition to some aliphatic CH carbon at 70 ppm. Amino acid analysis of the hydrolysate showed the presence of mainly glycine (1% of total weight)

with some NH_3, while ^1H NMR revealed the presence of small amounts of saturated by-products in addition to glycine (CH_2 at 3.83 ppm). Although hydrolysis resulted in a 20% drop in weight, the recovered hydrolysate only represented 7%, implying a loss of volatile material including NH_3, CO_2 and H_2O. An earlier gas chromatographic analysis[19] of 2N H_2SO_4 and 3N HCl hydrolysates did not show either xylose or any other identifyable sugar. These results were corroborated more recently by reports based on Curie point pyrolysis-mass spectrometry which showed little evidence for the presence of carbohydrate moieties[20] in melanoidins.

More information was obtained when ^{13}C and ^{15}N labeled melanoidins were acid hydrolyzed. Using ^{13}C labeled glycine, both hydrolyzed melanoidins B and A showed a decrease in all peaks originating from C-1, 172 ppm (Fig. 2, B4) and from C-2, 48.1; 31.2 and 22.5 ppm (Fig. 2, A4). Although Fig. 3.3 indicates no loss of material in the 20-50 ppm region and Fig. 2 A4 does, this loss is in effect scaled up by a large factor relative to the unlabeled case (Fig. 3.3). Vertical lines of spectra are not comparable. From a semiquantitative point of view, the loss in the melanoidin of C arising from C-2 of glycine is small compared to that of C arising from the sugar moiety. The water soluble materials showed peaks corresponding only to C-1 and C-2 of glycine. Surprisingly, other peaks were negligible, implying that C's originating from decarboxylated glycine, if released, were released in the form of volatile components. In the case of melanoidin C synthesized from D-xylose-1-^{13}C, only the peak at 68.8 ppm was reduced significantly (Fig. 2, C4). Hydrolysis did not affect the unsaturated C at 133 ppm. The corresponding ^{13}C NMR of the hydrolysate showed several sharp and several broad peaks over the entire spectrum, suggesting the presence of a polymer as well as some low molecular weight components. This fraction was not further investigated.

The ^{15}N CP-MAS NMR spectra of the ^{15}N labeled melanoidin E are shown in Fig. 5, A1 (CP=1 msec), B1 (CP=5 msec). As already reported[21] the melanoidin shows mainly amide N (≈100 ppm), with some amine N (≈22 ppm), pyrrole like N (≈130, 150 ppm) or imino N (≈150 ppm). A cross polarization time of 5 msec, Fig. 5 B1 allows one to better detect the atoms which cross polarize slowly, e.g., N

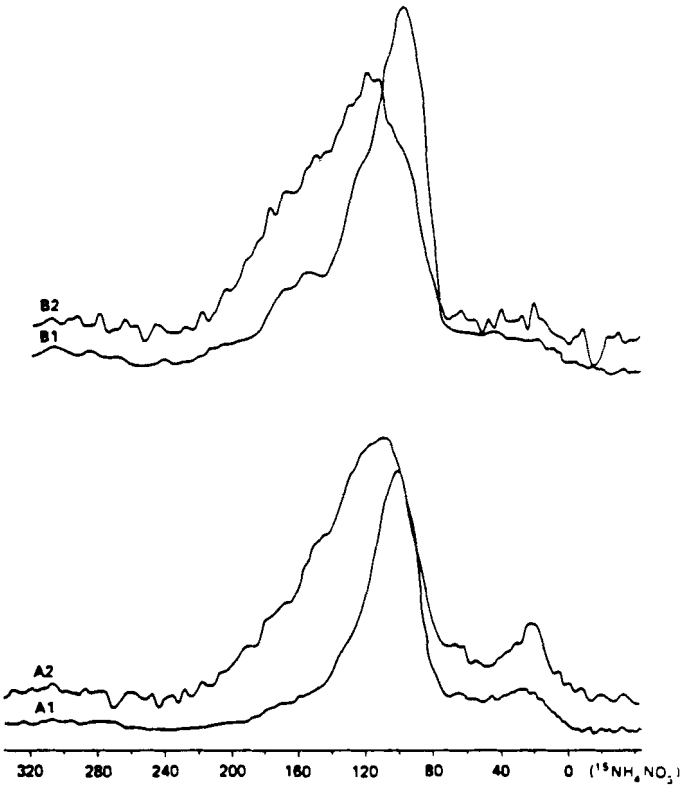

Fig. 5. ^{15}N CP-MAS NMR spectra of melanoidin E; before (A1, B1) and after 6N HCl hydrolysis (A2, B2). Spectra A1 and A2 were recorded using CP=1 msec, and B1, B2 using CP=5 msec.

without H atoms nearby. Major differences can be observed when comparing the latter spectra with those obtained after 6N HCl hydrolysis of the melanoidin (Fig. 5, A2 and B2). Although some of the amide peak still seems to be present (≈100 ppm, Fig. 5, B2), the main peak now appears downfield, at ≈123 ppm, the pyrrole N region of the spectrum. In addition, a broad peak further downfield (≈155) ppm, has also increased proportionaly. The ^{13}C and ^{15}N NMR spectra of melanoidin D and E treated with HCl at room temperature showed no visible changes.

In order to get a better estimate of what happened during hydrolysis of the ^{15}N labeled sample, as about the same amount of

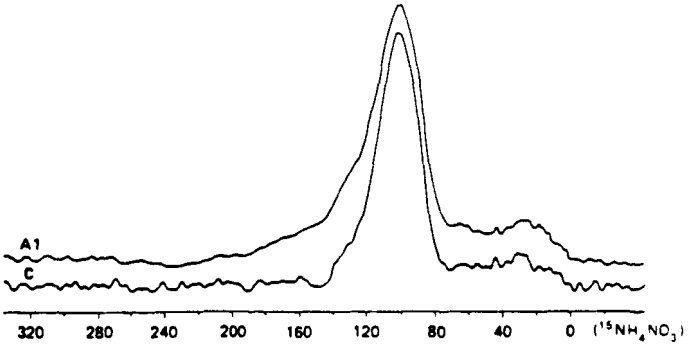

Fig. 6. ^{15}N CP-MAS NMR spectra of melanoidin E (A1). Spectrum C is difference between A1 and A2 (Fig. 4).

sample and the same number of scans were used for NMR before and after hydrolysis, spectrum A1 was scaled to A2 (same S/N). Fig. 6C, the difference between A1 and A2 is mainly composed of one symmetrical peak at ≈100 ppm (amide N), with a minor broad peak in the amine region (≈30 ppm) and the pyrrole region (130 ppm), the latter being possibly an artefact of subtraction.

The above results indicate that during hydrolysis, a) Cleavage of most of the amide linkage has occured as evidenced by the almost complete disappearance of the ≈100 ppm peak in ^{15}N NMR, corroborated by the greatly reduced amide I and II bands (1625 and 1550 cm^{-1}) in the DR-IR and the disappearance of the broad bands at 745 and 620 cm^{-1}. The presence of still a small amount of amide linkage after hydrolysis is not too surprising, in view of the fact that 20% of the amide linkages of Trisacryl GF 2000 resin resisted hydrolysis under the same reaction conditions. Trisacryl is a polymer containing four hydrophilic chemical groups per repeating unit (three hydroxy- methyl groups and on secondary amide group). This macro- molecule actually presents externally only hydroxymethyl groups, its polyethylene core and amide linkages are buried inside the coiling chain. b) Amine functions are produced as evidenced by the appearance of a small resonance at δ=30 ppm in the ^{15}N NMR spectrum. c) Most of the liberated amine functions, however, seem to have undergone subsequent reaction during the hydrolysis step, possibly yielding imine or pyrrole type N.

CONCLUSIONS

No definite structure can be put forward as a result of the above findings. Nevertheless, by using specifically labeled ^{13}C and ^{15}N reactants as well as the latest techniques for the study of insoluble polymers namely, ^{13}C and ^{15}N solid state nuclear magnetic resonance and diffuse reflectance infrared spectrometry, some progress was made towards the stuctural elucidation of the insoluble melanoidins obtained from D-xylose and glycine. The presence of amide linkages in melanoidins has been a matter of controversy. Rubinsztain et al.[22] reported that no amide linkages were found in melanoidins formed from amino acids and carbohydrates under basic conditions. Our earlier findings[21] as well as the results of this study, have definitely shown that in melanoidins obtained from 1M solution of xylose and glycine, the N is mainly in the amide form. These results agree with the recent results of another group[23,24] postulating that glycine was incorporated as the amide form into non-dialyzable melanoidin and oxidized melanoidins that had been prepared from a glucose-glycine system. Of the total glycine incorporated into the polymer, 50% was decarboxylated, probably the result of a Strecker degradation. No conclusions could be reached as to whether C-1 of glycine was incorporated into the polymer as a carboxyl C or an amide C or both. C-1 of D-xylose seems to be incorporated into the polymer mainly as a C bearing an OH function, and a smaller fraction as unsaturated C, probably the result of further dehydration.

EXPERIMENTAL

General Procedures

Glycine-1-^{13}C (90 atom % ^{13}C) and glycine-2-^{13}C (90 atom % ^{13}C) were purchased from MDS Isotopes, Division of Merck Frost Canada Inc., Montreal, Canada. D-xylose-1-^{13}C (99 atom % ^{13}C) was purchased from Cambridge Isotope Laboratories, Woburn, Mass. 01801. Unlabeled glycine was purchased from Sigma Chemical Company, St. Louis, MO 63178, and D-xylose from Pfanstiehl Laboratories, Inc.,

Waukegan, Ill. Trisacryl GF 2000 resin was from Fisher Scientific Ottawa, Ont. Canada. ^{1}H and ^{13}C solution NMR spectra were recorded on a Bruker WM 250 spectrometer. The ^{1}H spectra in D_2O were determined in 5 mm tubes, SI:16K; SW:3000 Hz, pulse delay: 5 sec. HOD (4.6 ppm) was used as reference peak. Bruker pulse program "Presat" was used to minimize the HOD peak. The ^{13}C NMR spectra were recorded in D_2O in 10 mm tubes, SI: 16K; SW: 15 KHz; pulse delay 10 sec.; 60° pulse.

^{13}C CP-MAS NMR spectra

The spectra of nonlabeled and ^{15}N labeled melanoidins (0.300 g) and ^{13}C labeled (0.400 g) were obtained at 45.78 MHz on a Bruker CXP-180 pulsed spectrometer with cross polarization and magic angle spinning as previously reported.[7,16] Cross polarization time: CP=1 msec; time between successive scans: 2 sec; number of scans: 5000 (non labeled and ^{15}N labeled melanoidins); 400 (melanoidins from 1-^{13}C and 2-^{13}C glycine); 800 (melanoidins from 1-^{13}C-xylose).

^{15}N CP-MAS NMR spectra

The spectra of the ^{15}N labeled melanoidins and Trisacryl resin were obtained at 18.25 MHz on the same spectrometer as previously reported.[19] Cross polarization times: 1 and 5 msec; recyling times 2 sec. Spinning rate: 3.4 KHz (delrin spinner); number of scans: 1500-2000 (^{15}N labeled melanoidins), 40000 (Trisacryl GF 2000).

Diffuse reflectance (DR) IR spectra

The spectra were measured with the use of a Digilab interferometer. A Globar source, and a medium-range (Υ min = 600 cm^{-1}) mercury, cadmium-telluride (MCT) detector were employed and the spectra were computed on the data system of a Digilab FTS-11 spectrometer. Melanoidins were pressed with KCl for one minute at a ratio of 1:200 (w/w). Spectra were measured at a nominal resolution of 4 cm^{-1}, and 250 scans were signal-averaged.

Melanoidin synthesis

Melanoidins were synthesized from molar solutions of D-xylose and glycine at 68°C. Sterile conditions were used throughout the syntheses and isolations. Initial carbohydrate and amino acid solutions were sterile filtered through 0.22 μm millipore filters. After six weeks of reaction time corresponding to the complete disappearange of xylose, the melanoidins were isolated by filtration, washed with double distilled water, sterile filtered until the filtrate was colorless and dried over P_2O_5. The filtrates and washings were combined and freeze dried.

Melanoidin A. Glycine-2-^{13}C (0.25 g) and glycine (0.5 g) was reacted with D-xylose (1.5 g) in water (10 ml). Yield (melanoidin A): 0.82 g. Yield (water soluble): 0.60 g.

Melanoidin B. Glycine-1-^{13}C (0.25 g) and glycine (0.5 g) was reacted with D-xylose (1.5 g) in water (10 ml). Yield (melanoidin B); 0.83 g. Yield (water soluble): 0.62 g.

Melanoidin C. D-xylose-1-^{13}C (0.25 g) and D-xylose (0.50 g) was reacted with glycine (0.375 g) in water (5 ml). Yield (melanoidin C): 0.42 g. Yield (water soluble): 0.31 g.

Melanoidin D. Glycine (0.75 g) was reacted with D-xylose (1.5 g) in water (10 ml). Yields: same as for A and B.

Melanoidin E. Glycine-^{15}N (1.5 g) was reacted with D-xylose (3.0 g) in water (20 ml). Yield (melanoidin E): 1.5 g. Anal. Found: C, 54.82; H, 5.42; N (Dumas), 6.53; N (micro-Kjeldahl), 6.6. Yield (water soluble): 1.2 g.

Melanoidin Degradation

NaOH hydrolysis of melanoidin E. Melanoidin E (0.300 g) in deoxygenated 1N NaOH (12 ml) was heated under N_2 at 50°C for 6 hrs. After cooling, the reaction mixture was neutralized with H_2SO_4 and dialyzed in tubing with MW cut off of 1000. The dialyzate was evaporated to dryness. Amino acid analysis revealed the presence of glycine (0.0008 g) and NH_3 (0.002 g). Weight of retentate: 0.297 g. Anal. Found: C, 54.73; H, 5.30; N (Dumas), 6.39; ash, 2.7.

HCl hydrolysis of melanoidin E. Melanoidin E (0.300 g) in 6N HCl (5 ml) was hydrolyzed in an evacuated sealed tube at 105°C for 18 hrs. After cooling, the reaction mixture was centrifuged, the precipitate washed with 6N HCl and H_2O until free of chlorine and dried over KOH. Weight: 0.240 g. Partial DR-IR: 1700, 1625, 1550, 1220, 745 and 620 cm^{-1}. Anal. Found: C, 53.75; H, 5.07; N (Dumas), 5.90; N (micro-Kjeldahl), 5.97.

The supernatant and washings were combined, evaporated under reduced pressure and dried over KOH. Weight: 0.022 g. Amino acid analysis showed the presence of glycine (0.003 g) and NH_3 (0.003 g). Dialysis of this soluble material in tubing with MW cut off of 1000, resulted in the retention of 10% of the material in the tubing. ^1H-NMR (D_2O): δ= 3.83 (s,CH_2).

HCl treatment of melanoidin E. Melanoidin E (0.003 g) was treated with HCl 6N at room temperature for 2 hrs, and worked up as above. ^{13}C and ^{15}N CP-MAS NMR spectra were the same as for the untreated polymer.

HCl hydrolysis of melanoidin D. Conditions and yields were the same as for melanoidin E.

HCl hydrolysis of melanoidins A, B and C. ^{13}C labeled melanoidins (0.150 g) in 6N HCl (5 ml) were hydrolyzed as described above. Melanoidin weight: 0.118 g. Soluble material: 0.016 g.

HCl hydrolysis of Trisacryl GF 2000. Trisacryl (0.7 g; N: 7.68%) was hydrolyzed and worked up using the same conditions as for the melanoidins. Weight (insoluble material): 0.281 g. Anal. Found: N, 2.09. Weight (soluble material): 0.476 g. Anal. Found: N, 9.08.

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