Contribution of Peptides to Volatile Formation in the Maillard Reaction of Casein Hydrolysate with Glucose

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The contribution of peptides to volatile formations was investigated by isolating the peptides from casein pancreatic hydrolysate and reacting them with glucose at 180 °C for 1 h and comparing them to the volatiles isolated from the original hydrolysate under the same conditions. Volatile flavor components from the model systems were isolated by simultaneous steam distillation and solvent extraction (SDE) and analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Strecker aldehydes and their corresponding substituted pyrazines were identified in the peptide fraction but at lower concentrations. Proline-specific Maillard reaction products were identified in both systems, and proline was found all in peptide forms by amino acid analysis. The results suggested that Strecker degradation of certain amino acids may proceed in spite of the blocked amino or carboxyl groups and that peptides contributed directly to the volatile formation by Maillard reaction.

INTRODUCTION

Due to consumer and market demands, enzymatic protein hydrolysates have become popular ingredients in the food and flavor industry. Compared to acid-hydrolyzed vegetable proteins (HVP), enzymatic protein hydrolysates contain a larger portion of peptides than free amino acids, owing to the high selectivity and specificity of proteases. However, information on the role of peptides in the formation of flavor compounds during thermal reaction is not as abundant as that on amino acids. In our previous experiments, we have shown that volatiles formed from the thermal degradation of cysteine and glutathione, the cysteine-containing peptide, in water (Zhang et al., 1988) as well as their interactions with 2,4-decadienal, glucose, or inosine 5′-monophosphate are significantly different (Zhang and Ho, 1989, 1991a,b).

Milk proteins and primary caseins have excellent nutritional value due to their physiologically well-balanced amino acid compositions. Hydrolysates of casein, which consist of small peptides and free amino acids, are commonly used in dietetic foods, in infant formula, and as a reactant in processed flavors. The chemical effects of peptides and amino acids on the formation of flavors, especially the Maillard products, were not studied in detail. The purpose of this study was, therefore, to study the contribution of peptides isolated from casein pancreatic hydrolysates (CPH) to the formation of reaction flavors.

EXPERIMENTAL PROCEDURES

Preparation of Casein Hydrolysate. Cow milk casein (prepared by Chemical Institute, Mongolian Academy of Sciences) was washed twice with distilled water to remove lactose. In a stainless steel reactor with a steam jacket and agitator, 15 kg of casein (dry base) was suspended in 150 L of distilled water. The suspension was heated to 75 °C by steam and 40% sodium hydroxide was used to dissolve the casein. Completely dissolved casein solution had a pH 7.5. The solution was then cooled to 45 °C by circulating cold water, and 3 kg of twice-minced bovine pancreas was added. The proteolysis was controlled at a constant temperature at 45 °C and pH 7.5 for 24 h. During the proteolysis, chloroform (100 mL) was added to prevent bacterial growth, and the enzymatic digestion of casein was terminated by adjusting the pH to 4.5 using concentrated hydrochloric acid.

A large volume of white precipitates was removed by filtration. Casein hydrolysate solution was then concentrated on a pilot vacuum evaporator at 65 °C. During this process, further precipitate was removed by another filtration. Finally, concentrated casein hydrolysate solution (35% solids) was adjusted to pH 7.5 and dried on a pilot spray drier under the following conditions: inlet temperature, 190 °C; outlet temperature, 90 °C The resulting casein pancreatic hydrolysate (CPH) was a fine powder of yellowish white color, with a good solubility of up to 40% in water.

Preparation of Peptide Fractions. A total of 45 g of casein hydrolysate was dissolved in 1800 mL of distilled water (2.5%). To this solution was slowly added 3600 mL of absolute ethanol, and white precipitates were formed at room temperature. The precipitates were collected by centrifugation at 3000 rpm for 10 min at room temperature and then dried in a vacuum oven at 25 °C. The yield of peptides (CPHP) was 3.3 g (7.3%).

Gel Chromatography. Sephadex G-25 (Pharmacia, Piscataway, NJ) was swollen and equilibrated in 0.05 M NaCl solution at pH 4.0. Column: i.d. = 0.85 cm; h = 60 cm; flow rate = 60 mL/h. CPH (54 mg), CPHP (29 mg), and tyrosine (5 mg) were each dissolved in 0.4 mL of 0.05 M NaCl solution (pH 4.0) and chromatographed under the same conditions.

UV absorption at 280 nm of each fraction was measured on a Hitachi UV-310 spectrometer, and chromatographic profiles of the samples were plotted against their elution volumes.

Reaction of Protein Pancreatic Hydrolysate and Peptide Fractions with Glucose at Elevated Temperature. Protein hydrolysate was washed with ethyl ether (1:20 w/v) for 2 h with vigorous shaking. After centrifugation, it was washed with methylene chloride (1:20 w/v) for 2 h with vigorous shaking. The solvent was removed by centrifugation, and the protein hydrolysate was air-dried

Three grams of protein pancreatic hydrolysate (CPH) and 3 g of its peptide fraction (CPHP) were dissolved in distilled water, and 1 g of glucose (anhydrous, reagent grade, Aldrich Chemical Co., Milwaukee, WI) was added to each sample solution.

The solution was adjusted to pH 7.5, and the volume was adjusted to 100 mL each. The reaction mixtures was transferred to a 0.3-L Hoke SS-DOT stainless steel sample cylinder, sealed, and heated in an oil bath at 180 °C for 1 h.

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Table I. Amino Acid Composition of Pancreatic Hydrolysate of Casein[a]

amino acid	free, mg/kg	bound (peptide), mg/g	amino acid	free, mg/g	bound (peptide), mg/g
Asp	11	61	Ile	8	29
Thr/Ser	14	65	Leu	39	
Glu	38	203	Tyr	19	3
Pro		250	Phe	25	
Cys			His	14	10
Gly/Ala	10	32	Lys	25	
Val	11	36	Arg	8	5
Met	9	11			

[a] Analysis was performed by K. M. Kludas, Institute of Vaccines, Dessau, Germany.

Distillation of Volatiles. The reaction mixtures were steam-distilled and extracted into methylene chloride by a Nickerson–Likens apparatus (Kontes, Vineland, NJ). An internal standard of tridecane (1.886 mg/mL) was added at a level of 235 ppm before steam distillation. The distillates were dried over anhydrous sodium sulfate and concentrated under a stream of nitrogen to a final volume of 0.2 mL.

Gas Chromatography. The gas chromatograph was a Varian Model 3400. A nonpolar fused silica capillary column [50 m × 0.32 mm (i.d.); 1.5-μm film thickness; HP-1 Hewlett-Packard] was used for this analysis. The column temperature was increased from 40 to 220 °C at a rate of 2 °C/min and was held for 30 min at the final temperature. The injector temperature was 270 °C, and the detector temperature was 290 °C. The flow rate was approximately 1 mL/min, the split ratio was 58:1, and the injected sample volume was 0.5 mL.

Quantitative determination was accomplished by internal standards previously mentioned. Linear retention indices for the volatile compounds were calculated versus *n*-paraffin standards (C_6–C_{22}, Alltech Associates, Deerfield, IL) as references (Majlat et al., 1974.)

Gas Chromatography–Mass Spectrometry. The high-resolution GC–MS consisted of a Varian Model 3400 gas chromatograph, a Finnigan MAT Model 8230 mass spectrometer, and a Finnigan SS 300 data system. The column and GC program were the same as described above. Mass spectra were obtained by electron ionization at 70 eV and a source temperature of 250 °C. The filament emission current was 1 mA.

RESULTS AND DISCUSSION

The amino acid composition of the casein hydrolysate listed in Table I was analyzed by K. M. Kludus of the Institute of Vaccine, Dessau, Germany. Results indicated that, due to the nature of enzymatic hydrolysis, 80% of the amino acid was still in bound peptide form. The hydrolysate was rich in proline, glutamic acid, aspartic acid, and valine; however, leucine, glutamic acid, phenylalanine, and lysine were the highest in free form and readily reacted. Sulfur-containing amino acids were relatively low in quantity. The discrepancy of this amino acid composition with that found in the literature (Adler-Nissen, 1986) might be reflected by the removal of precipitates during the preparation of hydrolysate and also by the different cow species from Mongolia.

Despite the relatively high enzyme/substrate ratio (1:5) and extended time of hydrolysis (24 h), the main products of the pancreatic digest of casein were peptides. Meanwhile, the diversity of peptides formed and their relatively low molecular weights made the isolation of peptides difficult, and only 7.3% yield of peptide precipitation by 66% ethanol was obtained.

The ethanol-precipitated fraction of casein hydrolysate was chromatographd on a Sephadex G-25 column in comparison with the original casein hydrolysate Comparison of elution volumes of this fraction with those of the original hydrolysate and tyrosine shows that this

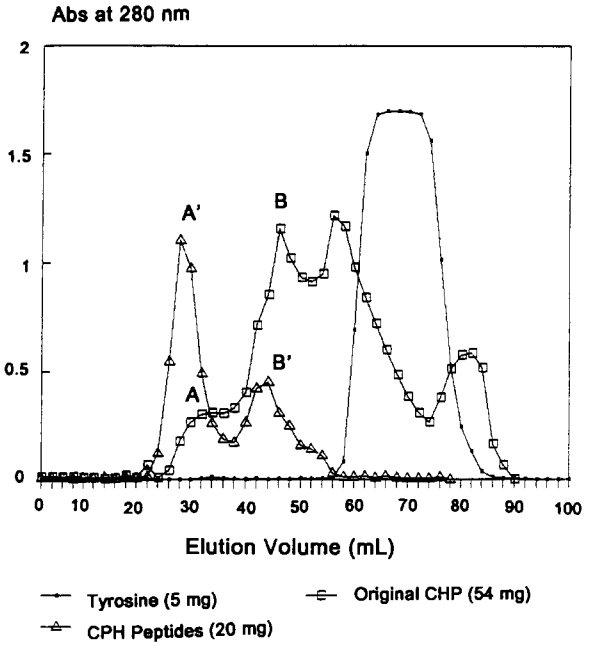


Figure 1. Elution profile of 66% ethanol-precipitated fraction (△) from casein pancreatic hydrolysate (□) against standard amino acid tyrosine (■) on a Sephadex G-25 column. Column: i.d. = 0.85 cm; *h* = 60 cm; flow rate = 60 mL/h; eluent 0.05 M NaCl, pH 4.0.

fraction consists of only relatively high molecular weight peptides (Figure 1).

As shown in Figure 1, we can assume that A′ and B′ constituents of the peptide fraction are A and B peaks in the chromatographic profile of the original hydrolysate. However, the ethanol-precipitated fraction of peptides was not homogeneous by molecular weight and consisted of at least two main components. The elution volume of tyrosine under the same conditions indicated that there were no substantial free amino acids present in the peptide precipitates.

The volatile compounds identified in the two model systems, casein pancreatic hydrolysate with glucose (CPH-G) and its peptide fraction with glucose (CPHP-G), are listed in Table II with their retention indices and quantitation. Compounds listed were identified by mass spectra and the Kovats indices (I_k) obtained from the HP-1 capillary column. Mass spectral data for the tentatively identified novel pyrazines are listed in Table III.

Besides the sugar-derived carbonyls, cyclotenes, and furans, the majority of the volatile compounds identified from the two model systems are Strecker aldehydes and their corresponding substituted pyrazines. Pyrazines are certainly not the only compounds produced by the model systems; however, the conditions selected seem to favor the formation of this group of compounds. A plausible mechanism for the formation of substituted pyrazines may be derived from the model experiments of Chiu et al. (1990) and the earlier work of Shibamoto et al. (1979). They suggested a pyrazine formation mechanism via a 2,5-dihydropyrazine intermediate. This intermediate was assumed from condensation of amino ketones, which were generated during Strecker degradations. Depending on different amino ketones, the intermediates range from none to three substituents before reacting with aldehydes. The intermediates either underwent dehydrogenation to form pyrazines or reacted with aldehydes to generate pyrazines with corresponding alkyl substitutions.

Listed in Table IV are the Strecker aldehydes and their corresponding alkyl-substituted pyrazines identified in the

Table II. Volatile Flavor Compounds Generated by Thermal Reaction of Casein Pancreatic Hydrolysate (CPH) and Its Peptides (CPHP) with Glucose at 180 °C and pH 7.5 for 1 h

compound	MW	I_k (HP-1)	amount, ppm CPH-G	amount, ppm CPHP-G
2-methylpropanal	72		56.98	39.18
2,3-butanedione	86	604	17.84	6.76
butanal	72	613	16.47	
3-methylbutanal	86	632	2911.52	248.03
2-methylbutanal	86	640	1073.52	154.02
2,3-pentanedione	100	662	0.32	23.39
pyrazine	80	706	428.60	74.44
pentanal	86	719	10.06	tr
2,4-pentanedione	100	724	24.00	5.20
dimethyl disulfide	94	733	51.35	10.27
2-methyltetrahydrofuran-3-one	100	773	211.19	107.26
4-methyl-3-penten-2-one	98	778		7.19
methylpyrazine	94	799	796.77	331.12
furfural	96	803	58.01	100.12
hexanoic acid	116	825	397.82	
2-acetylfuran	110	882	23.50	21.37
2,5-dimethylpyrazine	108	884	1100.54	248.33
ethylpyrazine	108	888		16.66
2,3-dimethylpyrazine	108	897	44.80	11.15
vinylpyrazine	106	905	8.86	
5-methyl-2-furfural	110	931	194.06	115.64
benzaldehyde	106	944	2.67	
dimethyl trisulfide	126	958	14.36	1.34
2-methyl-5-ethylpyrazine	122	973	50.34	11.65
2-methyl-6-ethylpyrazine	122	978	312.81	29.49
2-methyl-3-ethylpyrazine	122	983	39.17	9.21
2-methyl-5-vinylpyrazine	120	989	0.83	
2-hydroxy-3-methyl-2-cyclopentenone	112	1000	184.97	29.70
phenylacetaldehyde	120	1010	152.91	17.56
2-acetylpyrrole	109	1034	42.27	tr
2,5-dimethyl-3-ethylpyrazine	136	1058	59.72	6.85
2,6-dimethyl-3-ethylpyrazine	136	1064	24.00	5.95
2-methyl-5-propylpyrazine	136	1068	7.77	
5-methyl-2-formylpyrrole	109	1092	7.89	
2-hydroxy-3,5-dimethyl-2-cyclopentenone	126	1093	7.77	3.57
5-methyl-6,7-dihydro-5*H*-cyclopentapyrazine	134	1113	9.63	
2-[(methylthio)methyl]furan	128	1120	8.74	
2-methyl-5,6-diethylpyrazine	150	1130	4.83	
2-methyl-3,5-diethylpyrazine	150	1135	10.56	tr
2,5-dimethyl-3-propylpyrazine	150	1141	4.24	
isopentylpyrazine	150	1156	36.46	2.50
2-methyl-6,7-dihydro-5*H*-cyclopentapyrazine	134	1162	30.36	
2,5-dimethyl-3-isobutylpyrazine	164	1184	10.21	
2-methyl-5-isopentylpyrazine	164	1228	25.80	tr
2-methyl-6-isopentylpyrazine	164	1231	30.81	tr
2-methyl-5-(methylthio)furan	128	1256	3.61	
2,5-dimethyl-3-isopentylpyrazine	178	1296	52.91	1.23
2-hydroxy-3,5,5-trimethyl-2-cyclopentenone	140	1315	3.21	
5-acetyl-2,3-dihydro-1*H*-pyrrolizine	149	1359	29.21	2.48
2,3,5-trimethyl-6-isopentylpyrazine	192	1365	7.50	
[(methylthio)propyl]pyrazine	168	1386	1.23	
5-propionyl-2,3-dihydro-1*H*-pyrrolizine	163	1452	3.87	tr
5-acetyl-7-methyl-2,3-dihydro-1*H*-pyrrolizine	163	1500	3.37	tr
(phenylethyl)pyrazine	184	1552	5.28	
2-methyl-5-(phenylethyl)pyrazine	198	1623	15.67	
2,5-dimethyl-3-(phenylethyl)pyrazine	212	1685	17.34	

model systems, CPH-G and CPHP-G. Three major Strecker aldehydes identified were 2-methylpropanal, 3-methylbutanal, and phenylacetaldehyde, which were derived from their corresponding free amino acids: valine, leucine, and phenylalanine, respectively. As shown in Table II, these three Strecker aldehydes were the major volatile components for both CPH-G and CPHP-G model systems. This indicated that, in the presence of reducing sugars, the Strecker degradation of amino acids proceeded at elevated temperatures in spite of their peptide bondings, which was in agreement with the experimental results of Rizzi (1989). In his experiments, Rizzi (1989) heated fructose with different peptides, tripeptides, and mixtures of their corresponding amino acids. It was found that dipeptides containing valine and leucine produced significant amounts of Strecker aldehydes, 2-methylpropanal and 3-methylbutanal, despite the blocked amino group or carboxyl groups. However, in the present study, the ratio of the corresponding alkyl-substituted pyrazines to these Strecker aldehydes was only 1.2% in the peptide fraction (CPHP-G) compared to the ratio of 6.3% in the original hydrolysates (CPH-G). These results suggest that additional steps, such as hydrolysis of peptide bonds, might be involved in the Strecker aldehyde formation from peptides compared to that from free amino acids.

Another two Strecker aldehydes, acetaldehyde and methional, were not detected in the model systems, but their corresponding substituted pyrazines were identified. Acetaldehyde, having a low boiling point of 21 °C, might either be too volatile to be detected by methylene chloride extraction or be completely reacted with other components. Of the 16 alkyl-substituted pyrazines identified (Table IV), acetaldehyde was involved in 7, and their quantitation accounted for 73% of the total Strecker aldehyde substituted pyrazines. It is well-known that acetaldehyde could be formed from the Strecker degradation of alanine, cysteine, and cystine. It can also be formed with the fragmentation of deoxyhexosones during the Maillard reaction (Chiu et al., 1990). Therefore, certain portions of acetaldehyde could possibly come from sugar degradation during the Maillard reaction, and this may explain why alkyl-substituted pyrazines derived from acetaldehyde were so predominant in quantitation.

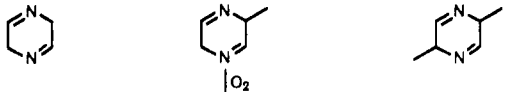
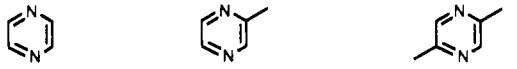
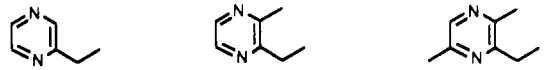
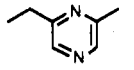
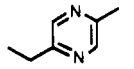
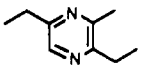
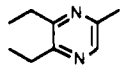
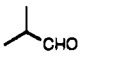
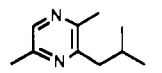
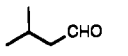
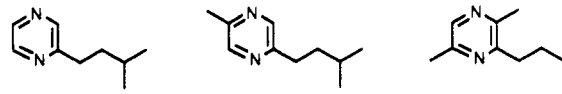
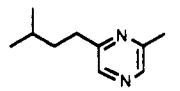
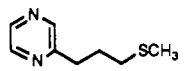
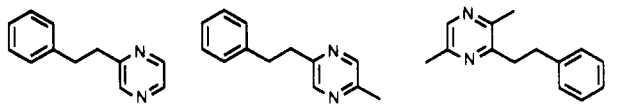
Peptides, on the other hand, have long been recognized as important compounds in processed flavors. The taste of peptides is important in cheese, meat, and soy sauce. Yamasaki and Maekewa (1978) isolated a delicious peptide from beef digested by papain and identified the primary structure of the peptide as Lys-Glu-Glu-Ser-Leu-Ala. However, reports of the contribution of peptides to volatile formation are lacking. Rizzi (1989), in his experiment, demonstrated that peptides could directly contribute to the volatile flavor componds instead of undergoing hydrolysis of peptide bonds to free amino acids. Numerous diketopiperazines were identified in cocoa bean extract, which were proposed to come from N-terminal peptides by heat treatment (Rizzi, 1989). Three proline-specific Maillard reaction products identified in this study were also directly formed from peptides. They are 5-acetyl-2,3-dihydro-1*H*-pyrrolizine, 5-propionyl-2,3-dihydro-1*H*-pyrrolizine, and 5-acetyl-7-methyl-2,3-dihydro-1*H*-pyrrolizine, which were identified in both CPH-G and CPHP-G. They were first identified by Tressl et al. (1985a,b) in a model system containing proline and reducing sugars. Later, Tressl et al. (1989) reported the occurrence of 5-acetyl-2,3-dihydro-1*H*-pyrrolizine in wort and beer, and this is the first report of these compounds occurring in a system containing a natural source of proteins.

The three proline-specific Maillard reaction products were found at higher concentration in the original hydrolysate (CPH-G) than in the peptide fraction (CPHP-G). Although proline was only found in peptide form when casein hydrolysate was subjected to amino acid composition analysis, it was difficult to tell whether proline was predominant in larger or smaller peptides. As shown by gel chromatography, the peptides prepared from CPH by 66% ethanol were primarily larger molecular peptides. It is possible that the formation of proline-specific Maillard products was due to reacting reducing sugar with small peptides, which were theoretically higher in CPH than in CPHP.

Table III. Mass Spectral Data of Alkylpyrazines Tentatively Identified from Reaction of Casein Pancreatic Hydrolysate (CPH) and Its Peptides (CPHP) with Glucose

alkylpyrazine	mass spectral data, *m/z* (relative intensity)
[(methylthio)propyl]pyrazine	168 (M^+, 0), 153 (20), 140 (10), 122 (19), 121 (85), 109 (16), 108 (11), 94 (13), 93 (16), 80 (29), 66 (12), 61 (100), 55 (181), 53 (28)
(phenylethyl)pyrazine	184 (M^+, 38), 183 (18), 169 (10), 115 (8), 107 (12), 91 (100), 77 (10), 66 (14), 65 (30), 51 (23), 43 (52), 39 (54)
2-methyl-5-(phenylethyl)pyrazine	198 (M^+, 45), 197 (29), 183 (22), 156 (5), 121 (20), 107 (7), 103 (5), 91 (100), 77 (12), 65 (32), 51 (14), 43 (23), 39 (50)
2,5-dimethyl-3-(phenylethyl)pyrazine	212 (M^+, 52), 211 (34), 197 (30), 135 (22), 121 (12), 108 (10), 107 (9), 91 (100), 80 (12), 65 (31), 51 (18), 42 (40), 39 (58)

Table IV. Pyrazines Formed from the Corresponding Strecker Aldehydes of Amino Acids in the Model System of Casein Pancreatic Hydrolysate (CPH) and Its Peptide Fraction (CPHP) with Glucose

Strecker aldehydes	sugar + amino acids or peptides or proteins	model system	
		CPH-G, ppm	CPHP-G, ppm
intermediate			
oxidation product		2325.9	635.9
CH_3CHO[a]		98.9	32.7
		312.8	29.5
		50.3	11.7
		10.6	tr[b]
		4.8	–[c]
		10.2	tr
		146.0	3.7
			
$CH_3SCH_2CH_2CHO$[a]		1.2	–
$C_6H_5CH_2CH_2CHO$		38.3	–
total		2999.0	731.5

[a] Strecker aldehyde not detectd. [b] Trace. [c] Not detected.

The major pyrrolizine, 5-acetyl-2,3-dihydro-1*H*-pyrrolizine, identified in the present systems was also found as the main product for the thermal reaction model systems containing dipeptides Pro-Gly or Gly-Pro with glucose at temperatures 130–180 °C. The production of this compound was dramatically reduced when the corresponding amino acid mixture was reacted with glucose under the same conditions (Oh et al., 1992).

In conclusion, experimental results suggested that peptides contributed directly to volatile formation under thermal reaction conditions, and certain amino acids in the bound form of peptides underwent Strecker degradation to form Strecker aldehydes, which contributed to the formation of corresponding alkylpyrazines in a Maillard reaction.

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