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Synthesis of Pyrazines Using Sugar Derived from Tobacco Cellulose and Hydrolyzed Tobacco F1 Protein as an Amino Acid Source *

by

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SUMMARY

An array of pyrazines have been synthesized using sugars derived from tobacco cellulose (CDS), ammonium hydroxide, and hydrolyzed tobacco F1 protein as a source of free amino acids (isolated amino acids from F1 hydrolysate, from filtered F1 hydrolysate and from non-filtered F1 hydrolysate). All reactions were performed at 120 °C for 60 min using a 40-mL Parr reaction vessel. Results showed that the addition of hydrolyzed F1 protein as free amino acid source increased the number of pyrazines with branched alkyl chains (for example, 2-butyl-3-methyl pyrazine) compared to when no amino acids were added. However, using isolated amino acids from hydrolyzed F1 protein versus just hydrolyzed F1 protein (filtered or not filtered) did not make a difference in yield or type of branched pyrazines. When non-filtered hydrolyzed F1 protein was used, the solution was much more viscous and contained suspended solid material when compared to the use of filtered hydrolyzed F1 protein. Addition of threonine (THR) to the reaction mixture did not increase the yield of pyrazines but did slightly shift the distribution of pyrazines toward those with three and four carbons attached. Similar but not identical arrays of pyrazines were obtained when somewhat resembling reaction conditions were applied on a larger reaction scale (~1.5 L).
A significant 50%-decrease in pyrazine yield was observed when the reaction temperature was reduced from 120 to 100 °C. No noticeable difference in the array of pyrazines from these two reactions was observed. In the majority of cases, the presence of free amino acids resulted in an increase in pyrazine yield coupled with a change in the qualitative array of pyrazines. These results clearly illustrate that sugar prepared from tobacco cellulose (glucose) can be used just like high fructose corn syrup to prepare flavor compounds via Amadori and Maillard reactions. The evidence highlights that hydrolyzed amino acids from F1 tobacco protein can be used via Maillard reactions to produce complementary arrays of pyrazine flavor compounds. [Beitr. Tabakforsch. Int. 28 (2018) 103–111]

ZUSAMMENFASSUNG

Unter Einsatz von Zuckern, die aus Tabakzellulose gewonnen wurden (CDS) sowie Ammoniumhydroxid und hydrolysiertem Tabakprotein F1 als Quelle freier Aminosäuren (isolierte Aminosäuren aus F1-Hydrolysat, aus gefiltertem F1-Hydrolysat sowie aus nicht gefiltertem F1-Hydrolysat) wurde eine Reihe Pyrazine synthetisiert. Alle Reaktionen wurden für eine Dauer von 60 min bei 120 °C unter Einsatz eines 40-mL Parr-Reaktionsbehälters durchgeführt. Die Ergebnisse zeigten, dass die Zugabe von hydrolysiertem Protein F1als Quelle freier Aminosäuren die Anzahl der Pyrazine mit verzweigten Alkylketten (z.B. 2-Butyl-3-methylpyrazin) gegenüber keiner Zugabe von Aminosäuren erhöhte. Jedoch ergab sich beim Einsatz von aus hydrolysiertem Protein F1 isolierten Aminosäuren im Vergleich

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zum bloßen Einsatz von hydrolysiertem Protein F1 (gefiltert oder nicht gefiltert) kein Unterschied hinsichtlich Ausbeute oder Typ der verzweigten Pyrazine. Wurde ungefiltertes hydrolysiertes Protein F1 eingesetzt, enthielt die Lösung Schwebstoffe und war sehr viel viskoser, als wenn gefiltertes hydrolysiertes Protein F1 verwendet wurde. Die Zugabe von Threonin zum Reaktionsgemisch erhöhte nicht die Ausbeute an Pyrazinen aber veränderte leicht die Verteilung der Pyrazine zugunsten von Pyrazinen mit drei oder vier Kohlenstoffatomen. Es ergaben sich ähnliche aber nicht identische Pyrazinreihen wenn vergleichbare Reaktionsbedingungen in einem größeren Reaktionsmaßstab (~1,5 L) angewandt wurden. Wenn die Temperatur von 120 auf 100 °C verringert wurde, konnte ein signifikanter Rückgang der Pyrazinausbeute um 50% beobachtet werden. Es wurde kein erkennbarer Unterschied in der Pyrazinreihe aus diesen beiden Reaktionen beobachtet. In den meisten Fällen führte das Vorhandensein von freien Aminosäuren zu einer Zunahme der Pyrazinausbeute gepaart mit einer qualitativen Veränderung der Pyrazinreihe. Diese Ergebnisse zeigen eindeutig, dass Zucker aus Tabakzellulose (Glukose) genauso wie Maissirup (HFCS, High Fructose Corn Syrup) eingesetzt werden kann, um mithilfe der Amadori-Umlagerung und der Maillard-Reaktion Aromaverbindungen herzustellen. Es wird anhand dieser Daten deutlich, dass aus dem Tabakprotein F1 hydrolysierte Aminosäuren eingesetzt werden können, um mittels der Maillard-Reaktion komplementäre Reihen an Pyrazin-Aromaverbindungen herzustellen. [Beitr. Tabakforsch. Int. 28 (2018) 103–111]

RESUME

Une série de pyrazines fut synthétisée à l'aide de sucres dérivés de la cellulose de tabac (CDS), d'hydroxyde d'ammonium et d'une protéine F1 de tabac hydrolysée en guise de source d'acides aminés libres (acides aminés isolés provenant de l'hydrolysat F1, de l'hydrolysat F1 filtré et de l'hydrolysat F1 non filtré). Toutes les réactions furent opérées à 120 °C durant 60 min dans un réacteur PARR de 40 mL. Les résultats révélèrent que l'ajout d'une protéine F1 hydrolysée en guise de source d'acides aminés libres augmentait le nombre de pyrazines à chaînes d'alkyles ramifiées (par exemple, de la 2-butyl-3-méthyl pyrazine) comparativement à l'absence d'ajout d'acides aminés. Toutefois, l'utilisation d'acides aminés isolés provenant d'une protéine F1 hydrolysée plutôt que seulement une protéine F1 hydrolysée (filtrée ou non) n'influença ni l'obtention ni le type de pyrazines ramifiées. Lors de l'utilisation d'une protéine F1 hydrolysée non-filtrée, la solution s'avéra bien plus visqueuse et contenait des matières solides en suspension contrairement à la solution obtenue grâce à la protéine F1 hydrolysée filtrée. L'ajout de thréonine au mélange réactionnel n'augmenta pas le rendement de pyrazines mais altéra légèrement la répartition des pyrazines vers celles ayant trois et quatre atomes de carbone. Des séries similaires, sans être identiques, de pyrazines furent obtenues lors de l'application de conditions de réaction relativement semblables à une échelle de réaction supérieure, à savoir ~1,5 L. Un écart significatif, à savoir une réduction de 50% du rendement des pyrazines, fut observé en cas de baisse de la température de réaction de 120 à 100 °C. Aucune différence notable dans la série de pyrazines obtenues par ces deux réactions ne fut observée. Dans la majorité des cas, la présence d'acides aminés libres provoqua une augmentation du rendement de pyrazines, associée à un changement dans la série qualitative des pyrazines. Ces résultats illustrent clairement que le sucre préparé à partir de cellulose de tabac (glucose) peut, à l'instar du sirop de maïs à haute teneur en fructose, entrer dans la préparation des composés aromatiques grâce à des réarrangements d'Amadori et des réactions de Maillard. Les preuves révèlent que les acides aminés hydrolysés provenant de la protéine de tabac F1 peuvent être utilisés, grâce aux réactions de Maillard, pour la production de séries complémentaires de composés aromatiques issus de la pyrazine. [Beitr. Tabakforsch. Int. 28 (2018) 103–111]

INTRODUCTION

Pyrazines can be formed by heating a mixture of sugars with nitrogen sources such as ammonium hydroxide (NH_4OH) and amino acids (AA) (1). When sugars and nitrogen sources, such as ammonium hydroxide (NH_4OH) and diammonium hydrogen phosphate ($(NH_4)_2HPO_4$), are employed in heated reactions, pyrazines are produced via Amadori reaction and when amino acids are involved pyrazines are produced via Maillard reaction. In most cases the structures of the pyrazines formed by Amadori and Maillard reactions are notably different. Using such an approach, pyrazine formation has been studied extensively (2–4). Most of these studies involved ideal pure reagent systems, in which specific sugars, such as glucose, fructose and or rhamnose, amino acids, such as threonine, leucine, isoleucine and alanine, and NH_4OH were examined for their effects on pyrazine yields and structures. As a result of these studies a considerable knowledge base exists concerning the effects of these different reagent variables as well as reaction conditions on pyrazine formation. It is also known that temperature, time, reagent ratios, and use of ammonium salts as additives, all have significant effects on pyrazine formation (1). Additionally, different amino acids are known to affect the structure of pyrazines formed (1, 4–6). Previously, an optimized method was developed to hydrolyze F1 protein for production of an array of tobacco-derived free amino acids (7). Tobacco F1 protein is considered to be the major protein fraction of the tobacco plant leaf comprising at least 2–5% by weight for cured leaf that usually has approximately 12% moisture. The F1 protein in tobacco is ribulose-1,5-biphosphate carboxylase/oxygenase and is commonly referred to with the abbreviation, RuBisCO. The composition (amino acid array) of this protein has been previously well defined and the types of amino acids therein are very suitable for participation in the Maillard reaction to produce pyrazines (7). Some of these F1 amino acids that participate in Maillard reaction are threonine, valine, alanine, leucine and isoleucine. The latter four of these amino acids are known for their capability for producing Strecker aldehydes that participate in the formation of pyrazines which usually produce pyrazines with alkyl side chains. These bear structures similar to those present in the free amino acid alkyl chains. Thus, these free

amino acids from F1 proteins can be envisioned to serve as nitrogen sources in reactions designed to prepare arrays of pyrazines.

In this current study, sugars derived from tobacco cellulose (CDS), hydrolyzed tobacco F1 proteins (isolated and purified free amino acids, filtered and non-filtered hydrolyzed F1 proteins), and NH_4OH were used as reagents to produce pyrazines. In all reactions, sugar concentration, NH_4OH volume, reaction temperature, reaction time, and final reaction volume were kept constant, employing a 40-mL Parr reactor. The yields and structures of the resulting pyrazines formed as functions of these variables were determined. Using the same conditions, reagent amounts and reaction volumes were scaled up to ~1 L and the pyrazines were isolated.

EXPERIMENTAL

Materials

Ammonium hydroxide (28–30%), d_6-methylpyrazine, ethanol, methanol and methylene chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). F1 protein was obtained from R.J. Reynolds Tobacco Co. (RJR) and hydrolyzed using previously developed optimum conditions (7). High fructose corn syrup (HFCS) was obtained from Ingredion (London, Ontario, Canada) as an aqueous solution containing ~70% sugar comprised of ~55% fructose and 45% glucose. Sugars derived from tobacco cellulose (CDS) were obtained from RJR and were comprised of an aqueous solution of ~70% glucose. Reversed-phase silica gel 90 C_{18} packing material was obtained from Sigma-Aldrich. Percent hydrolyzed amino acids in all of our hydrolyzed solutions were determined to be in the range of 50–55%, as previously reported (3, 7). All pyrazine synthetic reactions, with one exception, *vide infra*, were performed in a 40-mL Parr vessel which could handle high pressure and temperature reactions. In each reaction 7.7 g of CDS or HFCS were mixed with 1.8 mL of NH_4OH and then enough hydrolyzed F1 protein was added to make the mass of free amino acids equal to 0.4 g. For example, when 40 g of F1 protein were hydrolyzed in 1 L solution, the percent amino acids in solution was equal to 50%, which was equal to 20 g of amino acids in 1 L solution. In order to use 0.4 g of amino acids in a reaction, only 20 mL of the above solution was added to the reaction vessel. If the volume was less than 20 mL, enough H_2O was added to the solution to adjust the volume to a constant 20 mL. In some of the reactions, no hydrolyzed F1 protein was used and only 20 mL of water were added to adjust the volume. After completion of each reaction, the mixture was spiked with 250 µg of internal standard (d_6-methylpyrazine) and pyrazines extracted with 20–30 mL of dichloromethane. Each pyrazine we quantified against the mass of internal standard added to the extraction solvent. Reagent amounts and reaction volumes were proportionally increased for the reaction at ~1 L volume.

Different hydrolyzed F1 proteins as sources of amino acids were tested to determine which was better suited (highest yield, desired qualitative distribution) for formation of pyrazines using CDS. These hydrolyzed F1 proteins were:

1. Amino acids from hydrolyzed F1 protein which was filtered after hydrolysis
2. Amino acids from hydrolyzed F1 protein which was not filtered after hydrolysis
3. Amino acids which were isolated using resins after hydrolysis of F1 protein

Instrumentation

All GC/MS analyses were performed using a 6890 GC equipped with a 5973 Mass Selective detector (MSD) from Agilent (Wilmington, DE, USA). Separations were obtained using a DB-WAXETR capillary column (30 m × 250 µm I.D. with a film thickness of 0.25 µm) from J&W (Wilmington, DE, USA). The following operating parameters were used for each analysis:

· Injection port temperature	260 °C
· Purge valve	3 mL/min
· Purge time	1 min
· Total flow	24 mL/min
· Constant flow	1 mL/min
· Injection volume	2 µL, split 1:20
· Column oven initial temperature	50 °C
· Column oven initial time	3 min
· Column oven ramp rate	15 °C/min
· Column oven final temperature	250 °C
· Column oven final time	1 min
· MSD transfer line temperature	260 °C

MS Wiley library was used to identify each pyrazine. For quantitative analysis, pyrazines were quantified using single ion monitoring mode, employing the most intense ion for each specific pyrazine. Each pyrazine was quantified against the mass of internal standard (250 µg) added to the extraction solvent. Extracted ions which were used to quantify each pyrazine are listed in Tables 2–4.

RESULTS AND DISCUSSION

Reaction of HFCS with and without filtered hydrolyzed F1 protein

In the initial part of this study, HFCS was tested as the sugar base for the reactions to see what type of pyrazines were formed with and without amino acids from hydrolyzed F1 protein. Also, the mass of each pyrazine from each reaction (7.7 g of HFCS + 1.8 mL NH_4OH with and without 0.4 g of amino acids from hydrolyzed F1 protein at 120 °C for 60 min) was determined. Table 1 shows a list of all reactions and their conditions used in this study. Table 2 contains a list of detected pyrazines and their calculated mass based on addition of fixed mass of deuterated 2-methylpyrazine (internal standard) after each reaction (assuming all pyrazines have similar ionization response). It is important to note that values for pyrazine (m/z = 80) and methylpyrazine (m/z = 94) do not appear in the Tables. While they were a significant contributor to the pyrazine array in a number of these reactions (Figures 1–3), these pyrazines are generally recognized as having little sensory value coupled with less than optimum physical properties

Table 1. Reaction conditions and reagent ratios.

Reaction letter	Sugar source	Sugar mass (g)	NH_4OH mL	Amino Acids AA	Reaction temp. °C	Reaction time min	Special notes
A	HFCS	7.7	1.8	None	120	60	No mixing
B	HFCS	7.7	1.8	None	120	60	
C	HFCS	7.7	1.8	None	120	60	
D	HFCS	7.7	1.8	20 mL of filter F1 hydrolysate (1)*	120	60	0.4 g free AA
E	HFCS	7.7	1.8	2.5 mL of purified AA (3)	120	60	0.4 g free AA
F	CDS	7.7	1.8	None	120	60	
G	CDS	7.7	1.8	20 mL of F1 hydrolysate (1)	120	60	0.4 g free AA
H	CDS	7.7	1.8	2.5 mL of purified AA (3)	120	60	0.4 g free AA
I	CDS	7.7	1.8	5 mL of purified AA (3)	120	60	0.8 g free AA
J	CDS	7.7	1.8	20 mL of unfiltered hydrolysate (2)	120	60	0.4 g free AA
K	CDS	7.7	1.8	20 mL of F1 filter hydrolysate (1)	120	60	0.4 g free AA + 0.4 g of THR

* See Experimental section for the type of F1 hydrolysate was used.

Table 2. Mass of pyrazines formed via reaction of HFCS, NH_4OH and amino acids from filtered, unfiltered, and purified hydrolyzed F1 protein.

Retention time	Experiment conditions	Quantitative ion	A*** mass (µg)	B* mass (µg)	C* mass (µg)	D* mass (µg)	E*** mass (µg)
5.192	Pyrazine	80					
5.92	2-Methylpyrazine	94					
5.92	2-Methylpyrazine-d_6 (int. standard)	100	250.0	250.0	250.0	250.0	250.0
6.61	2,5-Dimethylpyrazine	108	2590.4	2172.6	1452.6	1454.5	1890.9
6.68	2,6-Dimethylpyrazine	108	9262.7	7980.5	4863.5	12314.2	14030.6
6.72	2-Ethylpyrazine	108	511.8	396.2	195.8	414.3	474.4
6.88	2,3-Dimethylpyrazine	108	1577.8	1343.7	999.7	1429.8	1607.7
7.29	2-Ethyl 6-methylpyrazine	122	140.3	102.9	46.7	165.4	176.9
7.37	2-Ethyl 5-methylpyrazine	122	43.3	29.9	15.4	22.9	28.9
7.52	Trimethylpyrazine	122	799.9	682.5	491.4	1011.7	1115.1
7.79	2-Vinylpyrazine	106	0.0	154.3	80.8	56.5	84.6
7.91	3-Ethyl-2,5-dimethylpyrazine	136	14.9	12.0	0.0	32.9	36.5
8.08	5-Ethyl-2,3-dimethylpyrazine	136	16.1	11.8	0.0	34.8	39.4
8.22	Tetramethylpyrazine	136	20.0	16.5	11.8	17.0	21.7
8.31	2-Ethenyl-6-methylpyrazine	120	41.7	31.5	16.1	26.9	33.0
8.42	2-Butyl-3-methylpyrazine	108	0.0	0.0	0.0	17.0	19.7
9.16	3-Methylbutylpyrazine	94	0.0	0.0	0.0	94.9	116.4
9.80	2,5-Diethyl-3-methylbutylpyrazine	122	0.0	0.0	0.0	53.6	60.1
	Total pyrazine mass (µg)		15087.2	12989.0	8192.7	17178.5	19781.0

*** single reaction, * duplicate reactions
A 7.7 g HFCS, 1.8 mL NH_4OH, No AA, 120 °C, 60 min (no mixing)
B 7.7 g HFCS, 1.8 mL NH_4OH, No AA, 120 °C, 60 min
C 7.7 g HFCS, 1.8 mL NH_4OH, No AA, 100 °C, 60 min
D 7.7 g HFCS, 1.8 mL NH_4OH, 20 mL F1 hydrolysate (0.4 g AA), 120 °C, 60 min
E 7.7 g HFCS, 1.8 mL NH_4OH, 2.5 mL purified AA (0.4 g AA), 120 °C, 60 min

(8, 9), and were hence not considered in the overall evaluations of the pyrazines produced as a result of these reactions. Noteworthy was the observation that butyl-substituted pyrazines were detected only in reactions that contained free amino acids from hydrolyzed F1 protein (see Table 2, reaction with and without AA). This influence on pyrazine structure due to the presence of free amino acids in model reactions is consistent with published results. Also, the total mass of pyrazines increased with addition of free amino acids from the hydrolysate. Results showed that at heating to 120 °C the reaction was more effective in the synthesis of pyrazines than at 100° C, in accordance with published results (4). In one reaction, the mixture was not stirred (no mixing), see Table 2, with minimal impact on pyrazine yield and qualitative array.

Results showed that the total mass of pyrazines for the non-stirred reaction was higher than for the reaction of those mixed. No clear rationale for this observation was readily apparent, and mixing/stirring was employed for all subsequent reactions. For HFCS, when purified amino acid was

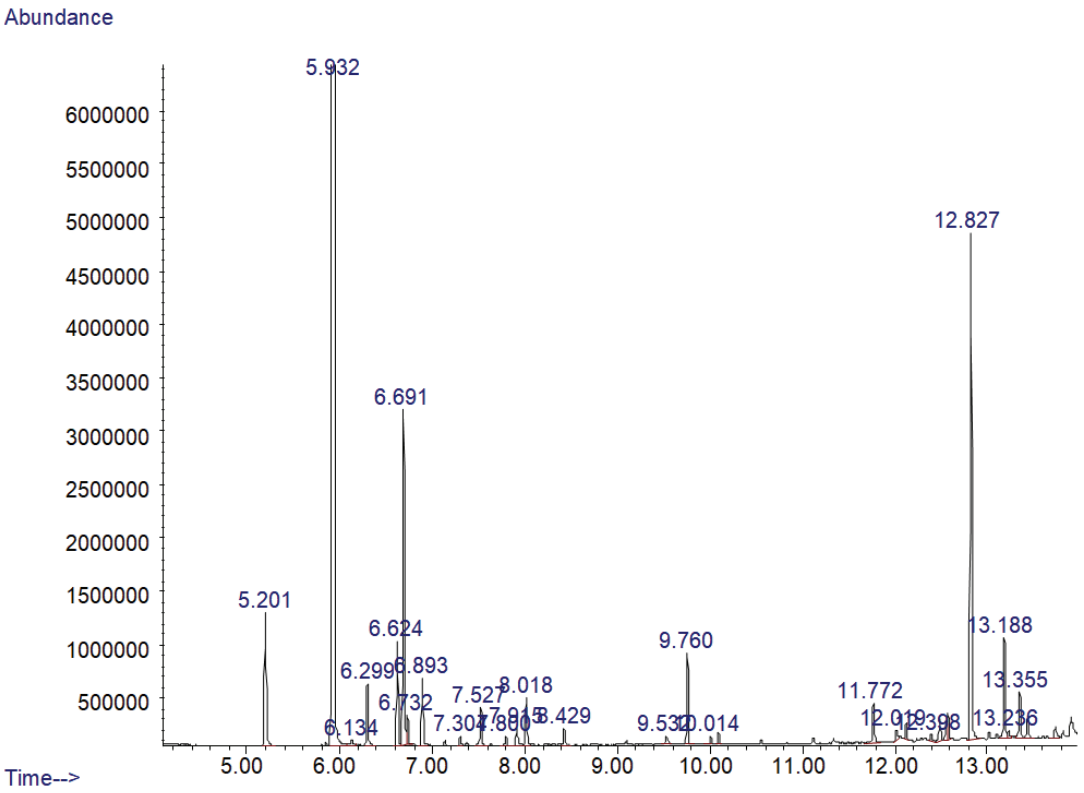

Figure 1. GC/MS of dichloromethane (DCM) extract from a reaction mixture of CDS + NH_4OH without any hydrolyzed F1 protein at 120 °C for 60 min.

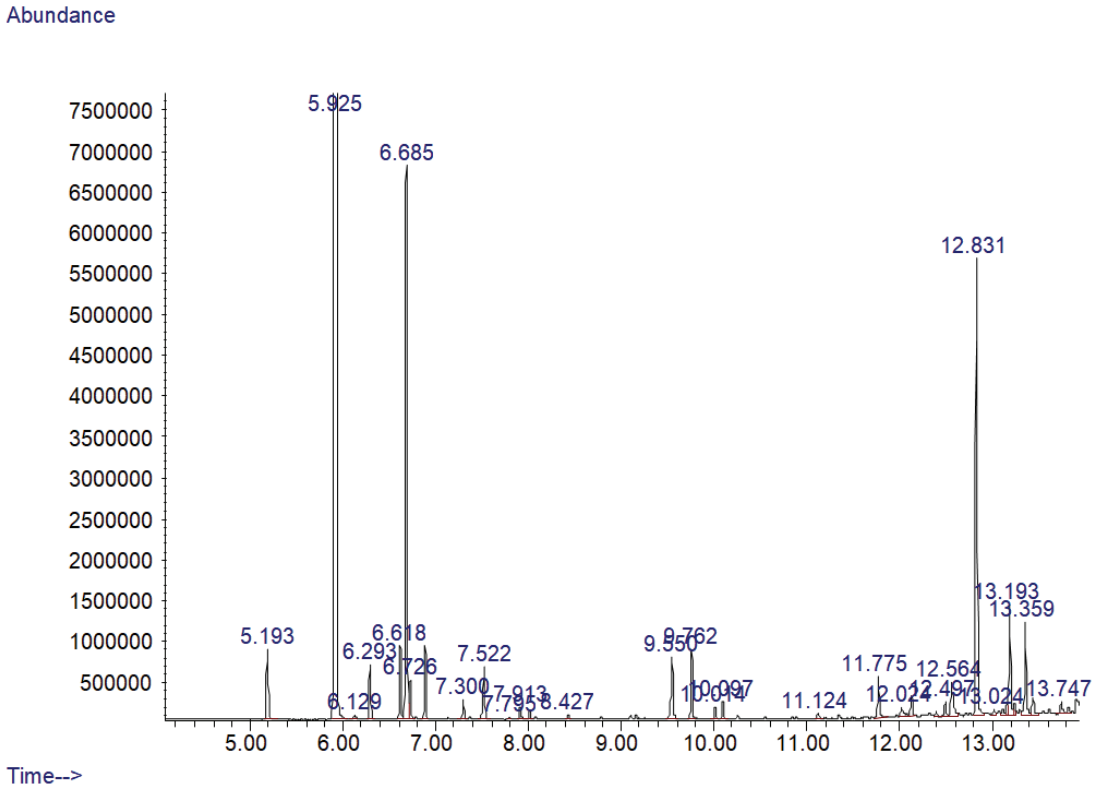

Figure 2. GC/MS of DCM extract from a reaction mixture of CDS (7.7 g) + NH_4OH (1.8 mL) + 0.4 g of AA's from filtered hydrolyzed F1 protein at 120 °C for 60 min. See Experimental section for chromatography conditions.

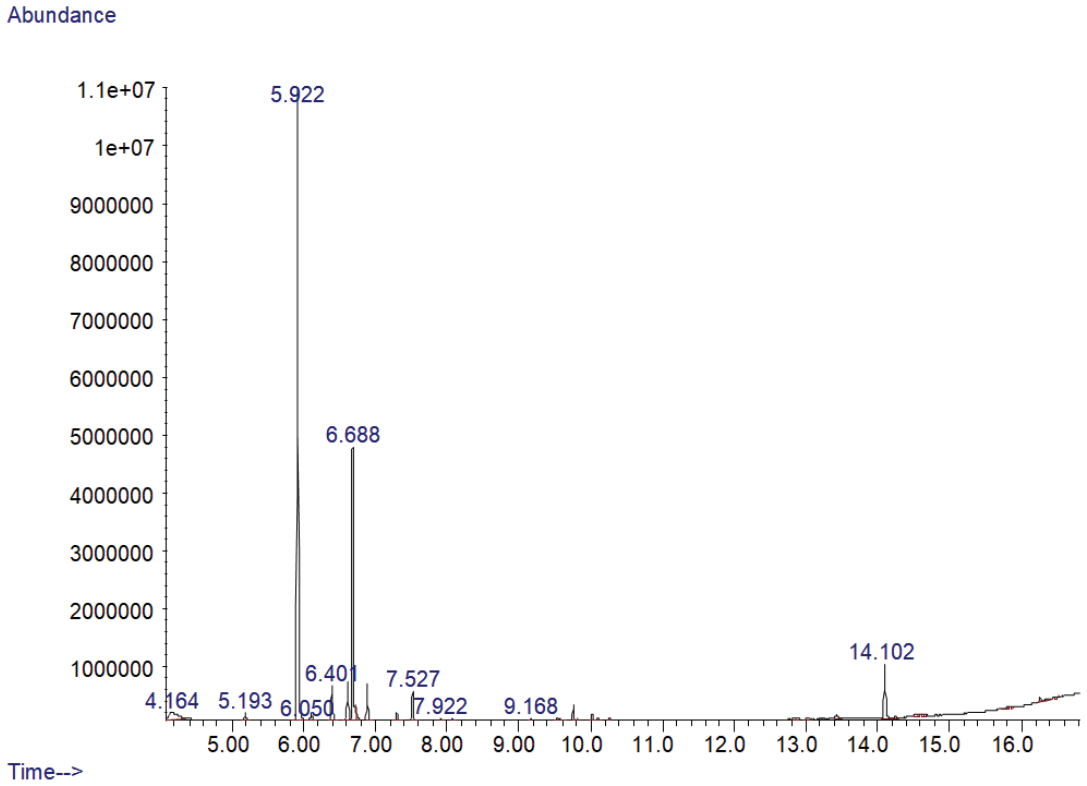

Figure 3. GC/MS of final pyrazines distilled and isolated from reaction of CDS with NH_4OH and hydrolyzed F1 protein in a Parr vessel at 120 °C for 60 min (see Table 4 for list of identified pyrazines).

used instead of filtered hydrolyzed F1 protein, the total mass of pyrazine was about 10% higher than with the filtered hydrolyzed F1 protein.

Reaction of CDS with and without filtered hydrolyzed F1 protein and isolated amino acids

In this part of the study, similar experiments as above were performed, but instead of HFCS as the sugar source, CDS was used. Reaction conditions were similar to those performed with HFCS. In this study, a constant mass (7.7 g) of CDS was reacted with a constant volume (1.8 mL) of NH_4OH, with and without 0.4 gram of F1 amino acids. The source of amino acids again was hydrolyzed F1 protein whose characteristics have been previously reported (7). Experiments were performed with and without filtered hydrolyzed F1 protein. Also, experiments were performed using 0.4 g purified amino acids after hydrolysis of F1 protein. In another experiment, the mass of amino acids was doubled to study the effect of additional amino acids on the synthesis of pyrazines. Also, in another experiment additional threonine (0.4 g) was added to the reaction that contained CDS, NH_4OH and 0.4 g of hydrolyzed F1 protein, see Table 1. This was done to test, if threonine could increase or decrease the mass of certain pyrazines and to test, if the presence of additional threonine would shift the distribution of the pyrazines toward those having three and four carbons attached, see Table 3.
As it was expected, addition of free amino acids caused pyrazines with larger than three carbon atoms (as a side chain) to be synthesized. Using isolated amino acids did not change the mass of pyrazines in the reaction. Also, using filtered or unfiltered hydrolyzed F1 protein did not change the yield of pyrazines in the reaction. However, it is important to note here that when filtered or isolated amino acids from hydrolyzed F1 protein were used, the resulting reaction solution was much cleaner and less viscous. Addition of extra threonine did not increase the yield of pyrazines, but a slight shift toward the distribution of those pyrazines having three and four carbons attached was observed, see Table 3. The presence of pyrazines having the 3-methylbutyl substituent strongly implicates the involvement of isoleucine in a Strecker/Maillard reaction pathway. For those reactions employing a reaction temperature of 120 °C, no significant differences were noted between the pyrazine yields when HFCS (fructose + glucose) and CDS (glucose) were employed. Figures 1 and 2 show chromatograms representative of the pyrazine profiles obtained with the smaller scale reactions.

Reaction of CDS with hydrolyzed F1 protein and ammonium hydroxide

In this part of the study, CDS was reacted with NH_4OH and optimized hydrolyzed F1 protein (7). The optimized F1 protein mixture was prepared by varying the type and amount of hydrolysis enzymes along with reaction conditions of temperature and time to produce the maximum amounts of free amino acids. In this reaction 323 g of CDS were mixed with 839 mL of hydrolyzed F1 protein and 76 mL of NH_4OH (employing the following reagent quantity adjustments: every 20 mL of hydrolyzed F1 pro-

Table 3. Average mass of pyrazines formed from reaction of CDS, NH_4OH and amino acids isolated from hydrolyzed F1 protein and amino acids from filtered and unfiltered hydrolyzed F1 protein.

Retention time	Experiment conditions	Quantitative ion	F* mass (µg)	G* mass (µg)	H* mass (µg)	I** mass (µg)	J** mass (µg)	K* mass (µg)
5.192	Pyrazine	80						
5.92	2- Methylpyrazine	94						
5.92	2-Methylpyrazine-d_6 (int. standard)	100	250.0	250.0	250.0	250.0	250.0	250.0
6.61	2,5-Dimethylpyrazine	108	2293.9	1534.8 (10.7)	1661.2	1595.1 (20.8)	2024.7 (6.1)	926.7
6.68	2,6-Dimethylpyrazine	108	7935.3	13745.8 (17.5)	11528.1	13791.8 (14.3)	13493.7 (6.6)	8381.7
6.72	2-Ethylpyrazine	108	455.6	535.6 (22.5)	518.3	671.3 (17.4)	670.7 (7.8)	962.7
6.88	2,3-Dimethylpyrazine	108	1380.9	1420.2 (3.3)	1379.5	1325.6 (14.0)	1667.6 (5.2)	987.6
7.29	2-Ethyl-6-methylpyrazine	122	118.6	212.5 (29.9)	187.1	351.3 (30.7)	246.8 (12,9)	491.8
7.37	2-Ethyl-5-methylpyrazine	122	36.4	31.1 (29.4)	31.0	48.8 (36.6)	44.9 (13.3)	49.0
7.52	Trimethylpyrazine	122	727.4	1023.4 (11.2)	970.2	1014.4 (18.9)	1127.8 (6.1)	922.8
7.79	2-Vinylpyrazine	106	179.4	71.8 (17.5)	98.4	59.1 (14.8)	104.3 (4.0)	46.7
7.91	3-Ethyl-2,5-dimethylpyrazine	136	11.0	36.0 (21.5)	34.0	52.6 (27.2)	33.4 (13.3)	83.2
8.08	5-Ethyl-2,3-dimethylpyrazine	136	15.5	41.4 (24.8)	38.6	56.9 (27.3)	43.2 (12.5)	95.7
8.22	Tetramethylpyrazine	136	18.9	20.1 (16.7)	21.3	21.0 (24.5)	24.4 (7.0)	16.4
8.31	2-Ethenyl-6-methylpyrazine	120	34.7	30.9 (31.9)	33.8	34.8 (14.8)	34.8 (7.0)	26.6
8.42	2-Butyl-3-methylpyrazine	108	0.0	20.3 (42.5)	20.3	28.3(25.8)	22.5 (15.5)	9.0
9.16	3-Methylbutyl pyrazine	94	0.0	147.0 (35.5)	166.8	273.2 (24.7)	139.1 (15.3)	82.6
9.80	2,5-Diethyl-3-methylbutyl pyrazine	122	0.0	32.5 (13.4)	56.4	92.1 (26.1)	43.0 (12.7)	33.0
	Total pyrazine mass (µg)		13265.2	18947.9 (13.5)	16787.7	19492.0 (15.8)	19791.1 (6.5)	13143.3

** Triplicate reaction (% RSD), * duplicate reactions
F 7.7 g CDS, 1.8 mL NH_4OH, No AA, 120 °C, 60 min
G 7.7 g CDS, 1.8 mL NH_4OH, 20 mL F1 hydrolysate (0.4 g AA), 120 °C, 60 min
H 7.7 g CDS, 1.8 mL NH_4OH, 2.5 mL purified AA (0.4 g AA), 120 °C, 60 min
I 7.7 g CDS, 1.8 mL NH_4OH, 5 mL purified AA (0.8 g AA), 120 °C, 60 min
J 7.7 g CDS, 1.8 mL NH_4OH, 20 mL unfiltered F1 hydrolysate (0.4 g AA), 120 °C, 60 min
K 7.7 g CDS, 1.8 mL NH_4OH, 20 mL F1 hydrolysate (0.4 g AA), 120° C, 60 min. plus 0.4 g THR

tein contained 0.4 g of F1 amino acids and every 7.7 g of CDS required 1.8 mL of NH_4OH). The mixture was prepared in a 1.5-L glass container which was housed in a Parr reaction vessel with mixing device and thermometer. The mixture was stirred and heated for 40 min until the temperature of the reaction mixture was stable at 120 °C. After stabilization of the temperature at120 °C, the mixture was stirred and heated for additional 60 min. These reaction conditions were very similar to reaction J, with a longer overall reaction time being employed. Then the reaction mixture was cooled and transferred into a glass jar container and kept in a refrigerator for later steps which included distillation and isolation of pyrazines.

Distillation of pyrazines from large reaction of CDS

Due to the relatively large volume of the reaction mixture (approximately 1.1 L) and a limited-sized distillation apparatus, only 350–400 mL of the reaction mixture was distilled each time. The mixture was heated to 120 °C with an oil bath and the distilled material was collected in a clean flask in a manner previously described (10). After collection of approximately 65–75 mL of distilled solution in a clean flask, an analysis of a dichloromethane (DCM) extract of the reaction mixture confirmed a significant reduction of the amount of pyrazines remaining in the reaction mixture. This process was repeated two more times with the remaining undistilled reaction mixture to isolate all pyrazines. The final volume of all distilled material from 1.1 L reaction mixture, which contained only pyrazines and water, was approximately 200–220 mL of a colorless solution.

Next, a glass column (60 × 5 cm) packed with only 15 cm (about 85 g) of C_{18} packing (SPE cartridges packing material from Supelco (Bellefonte, PA, USA) was used to isolate the pyrazines, loading ~30–35 mL of distillate per separation. No optimization was performed on how much of the distilled solution could be loaded onto the column before overloading it. The only test to show that the column was not overloaded, was to make sure that any distillate eluting from the column did not contain any pyrazines. This test was carried out by a simple DCM extraction of eluted H_2O from the column followed by analysis via GC/MS.

Before loading the column with the distillated solution, the column was conditioned first by washing with 150–200 mL of methanol (MeOH) through the column, followed by washing with150–200 mL of 0.1% formic acid solution and finally washing it with 100 mL of deionized H_2O. After the water was eluted from the column without letting the column go dry, 30–35 mL of distilled solution was loaded onto the column and allowed to drain at a flow rate of 3–5 mL/min. Afterwards the column was washed with an additional 75–100 mL of deionized H_2O. At this point all eluted water from the column was collected and the solution was analyzed indicating no detectable pyrazines. After complete elution of H_2O from the column, it was dried for 5–10 min by passing N_2 through it. Finally, all

trapped pyrazines were eluted with ethanol or methanol. Due to lower polarity of ethanol, a larger volume (135–150 mL) of ethanol was required compared to methanol (100–120 mL) to elute the pyrazines from the C_{18} column. Figure 3 illustrates the GC/MS of the isolated pyrazines that were distilled from the reaction of CDS with NH_4OH and hydrolyzed F1 protein at 120 °C in a Parr vessel for 60 min, afterwards they were isolated and then eluted from the C_{18} column. Table 3 shows list of identified pyrazines in Figure 3 and their retention times. It is important to note that even though their presence is not clearly indicated by Figure 3, all of the pyrazines noted in Table 4 were positively identified in the isolate. When qualitatively comparing the array of pyrazines produced by employing the small reaction volume with the array obtained by employing the relatively large reaction volume, differences could be detected.

Table 4. List of identified pyrazines in Figure 3.

Retention time	Compound	Quantitative ion
5.193	Pyrazine	80
5.922	Methylpyrazine, + int. standard	94, 100
6.620	2,5-Dimethylpyrazine	108
6.688	2,6-Dimethylpyrazine	108
6.726	Ethylpyrazine	108
6.890	2,3-Dimethylpyrazine	108
7.304	2-Ethyl-6-methylpyrazine	122
7.369	2-Ethyl-5-methylpyrazine	122
7.527	Trimethylpyrazine	122
7.924	3-Ethyl-2,5-dimethylpyrazine	136
8.084	2,3-Dimethyl-5-ethylpyrazine	136
8.220	Tetramethylpyrazine	136
9.169	2-Pentylpyrazine	94
9.580	2-Isopentyl-3-methylpyrazine	108
9.816	2,5-Dimethyl-3-isoamylpyrazine	122
10.104	1-(6-Methyl-2-pyrazinyl)-1-ethanon	122

Specifically, the last four pyrazines listed in Table 4 (large reaction volume) fail to appear anywhere in the list of pyrazines found stemming from all the smaller reaction volume reactions. A possible reason for this discontinuity in qualitative arrays could rest with the sample preparation approaches which were very different. For all of the small volume reactions, DCM extraction of the reaction mixture was employed to determine the pyrazine array. For the relatively large reaction volume, the pyrazines were first steam-distilled from the reaction mixture and the clear colorless aqueous distillate was extracted with DCM. It could be speculated that differences in steam volatility precipitated a difference in the pyrazine array, that is, some of the pyrazines present in the larger reaction volume might not be significantly volatile with steam, yet readily extractable with DCM. Steam distillation is a convenient method for segregating the desirable pyrazines away from less than desirable side reaction compounds, contained within the reaction mixture.

CONCLUSIONS

The results of this investigation demonstrated that the addition of enzymatically hydrolyzed tobacco F1 protein as a source of free amino acids could increase the yield of pyrazines with more than three carbons attached to the pyrazine ring compared to the pyrazine yields when no free amino acids were employed. This is consistent with well known amino acid Maillard and Amadori chemistry. Also, it was shown that use of isolated amino acids from enzymatically hydrolyzed tobacco F1 protein as the amino acid source did not result in increased yields or increased types of pyrazines synthesized when compared with the results obtained when the hydrolysis reaction mixture was employed. Also, there was no difference in yields or type of pyrazines whether filtered or unfiltered hydrolyzed F1 protein was used in the reaction mixture. Addition of threonine did not increase yields or type of pyrazines in the synthesis but it did slightly shift the pyrazine distribution toward those with three and four carbons attached. After distillation of the pyrazines from a relatively large 1 L-reaction, followed by isolation of the pyrazines using column chromatography packed with C_{18}, pyrazines having alkyl groups numbering between two and five carbons were produced. Thus, free amino acids generated by enzymatic hydrolysis of tobacco F1 protein and sugar (glucose) from tobacco cellulose can serve as nitrogen and carbon sources, respectively, for the synthesis of a unique array of pyrazines, known for their positive sensory attributes. The sugar (glucose) derived from tobacco was as effective as HFCS in the production of pyrazines, resulting in a qualitatively similar array of pyrazines as well. A reaction temperature of 100 °C produced approximately 50% less pyrazines when compared with to a reaction temperature of 120 °C.

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