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Formation of N-Nitrosopyrrolidine in Fried Bacon

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ABSTRACT

Formation of N-nitrosopyrrolidine (N-Pyr) from proline and N-nitrosoproline (N-Pro) was investigated by cooking pork slices to which these precursors had been added. When the cooking system was heated in an oil bath at 200 C for 12 min, 0.33 and 2.18% yields of the N-nitrosamine were obtained from proline and N-Pro, respectively. The N-Pro contents of pork slices to which two levels of nitrite were added were determined after 1 and 8 days of storage at 2 C. Results indicate that the formation of N-Pro occurs at such a rate that 1 day after addition of nitrite there is theoretically sufficient N-Pro formed to account for the quantities of N-Pyr isolated from cooked bacon. However, the rate of decarboxylation of the initial N-Pro in raw bacon is not great enough to account for the N-Pyr isolated from cooked bacon.

Recent research has indicated that N-nitrosopyrrolidine (N-Pyr) occurs in fried bacon but not in other cured meat products (*6,7*). It is also very evident that N-Pyr formation in bacon depends on temperature stresses and the nature of these stresses. This is supported by the fact that no N-Pyr has been isolated from raw bacon (*8*). The presence of N-Pyr in fried bacon and the cooked-out fat has aroused considerable interest as to its mode of formation and consequently various precursors have been suggested, including proline (*2,4,5,12,16*), collagen (*12,16*), spermidine (*15*), and putrescine (*2,16,20*).

Free proline appears to be the most probable precursors of N-Pyr in bacon. However, the mechanism of N-Pyr formation has not yet been clearly defined. Several pathways have been proposed. Sen et al. (*23*) proposed that free proline in bacon is converted to N-nitrosoproline (N-Pro) which when subjected to thermal stresses is decarboxylated to N-Pyr. These findings are consistent with the data of Hwang and Rosen (*17*). Nakamura et al. (*21*) showed that the mechanism involved depends on the cooking temperature. In the temperature range of 100-150 C, amounts of N-Pyr formed from free proline via pyrrolidine were almost similar to those formed via N-Pro. At temperatures above 175 C, the yield of N-Pyr via pyrrolidine was greater than that formed via the N-Pro pathway.

This study was designed to evaluate the contribution of proline and N-Pro to the formation of N-Pyr under actual conditions of frying bacon. The formation of N-Pro in pork belly slices treated with two levels of nitrite (150 and 1000 ppm) was also investigated.

EXPERIMENTAL PROCEDURES

Formation of N-Pro

N-Pro was prepared according to the procedure of Hansen et al. (*14*). Melting point determination and thin layer chromatographic analysis using a solvent system of 95% ethanol-benzene-water (4:1:1) were used for confirmation of purity of the N-nitroso derivative.

Formation of N-Pyr in nitrite-treated pork samples

Pork bellies obtained 24 h after slaughter from a commercial processor were skinned, quartered, and cut in thin slices (1/8 inch). These slices were freeze-dried for 24 h in a Virtis RePP Model No. 42 sublimator at a pressure of 5 μ and a shelf temperature of 24 C. Moisture loss was recorded. The slices were then rehydrated to their original moisture levels using aqueous solutions containing sodium nitrite, proline, or N-Pro, as required. The concentrations of these solutions were calculated so as to give the range of N-Pyr precursor concentrations as indicated in the text.

The amount of N-Pyr formed as a result of cooking the pork slices was determined using the heating system previously described (*10*). A 100-g sample of the rehydrated pork slices containing the added precursor (s) was cut into small pieces and placed in a two-necked, 500-ml round bottom flask fitted with a distillation head, condenser, and receiving flask. A thermometer was placed in the second neck to record the maximum temperature of the flask contents reached during cooking. The flask was heated in an oil bath and the contents of the flask were continuously stirred during the heating period by means of a magnetic stirrer. At the completion of the cooking period, the condenser and distillation head were washed with distilled water and the washings and condensate added back to the original heating flask. The contents of the flask were then steam distilled after the addition of 300 ml of 3 N NaOH.

The distillate (350 ml) was extracted with 3 × 100-ml aliquots of dichloromethane and the combined solvent extracts washed successively with 50-ml aliquots of 6 N HCl and 5 N NaOH. After drying over anhydrous Na_2SO_4, the solvent extract was concentrated to a volume of 2.5 ml in a Kuderna-Danish concentrating apparatus. Hexane (1 ml) was added and the extract further concentrated under a stream of nitrogen to a volume of 1 ml. The solvent concentrate was cleaned up and analyzed for its N-Pyr content using the thin-layer

chromatographic method previously described by Gray and Dugan (*12*).

Isolation of N-Pro from nitrite-treated pork slices

The procedure of Nakamura et al. (*21*) was essentially used. Presence of N-Pro in the nitrite-treated pork slices was established by converting it to its methyl ester and confirming by mass spectrometry. Samples were stored at 2 C and N-Pro analyses were conducted after 1 and 8 days of storage.

Gas chromatographic analysis

A Hewlett-Packard 6720A dual column gas chromatograph equipped with flame ionization detectors and stainless steel columns (10 ft × 1/8 inch o.d.) packed with 6% Carbowax 20M on 80-100 mesh Chromosorb W was used for the analysis of N-Pyr. The chromatograph was temperature programmed from 80 to 180 C at 10 C/min. The carrier gas flow rate was 21 ml/min.

The analysis of N-Pro methyl ester was carried out using columns of 3% SP2100 on 80-100 mesh Chromosorb W. Temperature programming was in the range of 100 to 200 C, at 5 C/min.

Gas chromatography - mass spectrometry

Mass spectra were obtained using a combined GLC-mass spectrometer LKB 900 equipped with a glass column (6 ft × 1/8 inch o.d.) of 3% SP2100 with an ionizing energy of 70 eV. The spectra were reported as bar graphs by means of an on-line data acquisition and processing program (*25*).

Analysis of bacon for nitrite and moisture contents

Residual nitrite was determined by the A.O.A.C. procedure (*1*). Moisture contents were obtained by drying ground samples at 100 C for 24 h.

RESULTS AND DISCUSSION

Four commercial samples of bacon were purchased in a local retail store and analyzed for their moisture and nitrite contents (Table 1). Average nitrite levels for the lean and adipose tissues were 43 and 25 ppm, respectively, while the average moisture contents were 67.5 and 11.9%, respectively. Assuming that all the nitrite was dissolved in the water, concentrations of residual nitrite in the aqueous phases of the lean and adipose would be 63.7 and 210 ppm, respectively. A previous study (*9*) has shown that the average free proline contents in five green pork bellies stored at 2 C for 8 days were 24.1 μM/100 g of lean tissue and 10.5 μM/100 g of adipose tissue. From the moisture contents obtained in this study, and assuming that most of the free proline is dissolved in the water, the aqueous phases of lean and adipose tissues would contain 35.7 and 88.2 μM proline/100 g of tissue, respectively. Thus it would appear that conditions are favorable in both the lean and adipose tissues for N-Pro formation, although the latter seems the more probable location because of the higher concentration of nitrite. This study was designed to quantitate the presence of N-Pro in pork slices treated with nitrite.

Two levels of nitrite were used; 150 ppm which is the legal amount permitted in the curing of side bacon in Canada and 1000 ppm which was used to enhance formation of N-Pro (Table 2). Results indicate that formation of N-Pro occurs at such a rate that 1 day after addition of nitrite, there is sufficient N-Pro formed to account for the quantities of N-Pyr isolated from cooked bacon provided the percent conversion of N-Pro to N-Pyr during frying is sufficiently great. The N-Pro content increased slightly during storage at 2 C for 8 days, although sample 4 doubled its N-Pro content during this storage period. The values reported were based on a 50% recovery and were much lower than those reported by Kushnir et al. (*19*). These authors reported values ranging from 0.38 to 1.18 ppm for three bacon samples and these values were not corrected for losses incurred during the extraction procedure. Nakamura et al. (*21*) analyzed five bacon samples, both raw and fried, and failed to detect any N-Pro. Ivey (*18*) reported that bacon cured in brines containing 1600 ppm of nitrite and having a residual nitrite content of 100 ppm contained greater than 100 ppb of N-Pro. Frying of the bacon reduced the N-Pro concentration by 86-100%. As expected, the higher level of nitrite used in the present study produced greater amounts of N-Pro.

The precursor role of proline and N-Pro generally has been studied in model systems simulating the pan-frying of bacon. Many of these studies have been conducted in the dry state (*5,12,16*) and in oil systems (*2,12,23*). In this study, the contribution of proline and N-Pro to formation

TABLE 2. *Effect of nitrite level on formation of N-nitrosoproline in pork slices.*

Carcass number	Nitrite added (ppm)	N-Nitrosoproline (ppb)[a,b]	
		Day 1[c]	Day 8[c]
1	150	101	115
2	150	78	92
3	150	43	61
4	150	210	416
1	1,000	340	389
2	1,000	243	430
3	1,000	782	913
4	1,000	833	1,083

[a]Calculations based on 100-g pork sample.

[b]Limit of detection, 20 ppb.

[c]Day 1 and Day 8 refers to the number of days of storage at 2 C after the addition of nitrite.

TABLE 1. *Analysis of four commercial bacon samples for moisture and nitrite contents.*

Sample number	Percent composition of sample		Percent moisture in			Nitrite content (ppm)	
	Lean	Adipose	Whole	Lean	Adipose	Lean	Adipose
1	58.1	41.9	41.2	63.8	13.8	48	28
2	45.1	54.9	37.0	69.2	11.3	34	31
3	42.3	57.7	36.3	65.9	12.8	61	30
4	50.6	49.4	41.9	71.2	9.8	28	12
Average	49.0	51.0	39.1	67.5	11.9	43	25

of N-Pyr was evaluated by frying pork slices containing the added precursors. The cooking was done in an all glass distillation system so that the volatilized N-Pyr could be collected to quantitate the total N-Pyr produced during the cooking of the pork slices. Tables 3 and 4 show that when the cooling system was heated in an oil bath at 200 C for 12 min, 0.33 and 2.18% yields of N-Pyr were

TABLE 3. *Effect of temperature on formation of N-nitrosopyrrolidine in fried pork slices containing 1 mM N-nitrosoproline, time of nitrite, time of heating, 12 min.*

Temperature of oil bath	Temperature (max) of cooking system (C)	N-Nitrosopyrrolidine[a,b,c] (mg)	Percent conversion Pro → N-Pyr
100	92	ND	—
120	104	0.02	0.02
140	125	0.08	0.07
160	149	0.16	0.13
180	172	0.24	0.21
200	189	0.38	0.33

[a]Average of duplicate determinations.
[b]Uncorrected for contribution from naturally occurring free proline in the pork slices.
[c]Confirmed by mass spectrometry.

TABLE 4. *Effect of temperature on formation of N-nitrosopyrrolidine in fried pork slices containing 1 mM N-nitrosoproline, time of heating, 12 min.*

Temperature of oil bath (C)	Temperature (max) of cooking system (C)	N-Nitrosopyrrolidine[a,b] (mg)	Percent yield N-Pro → N-Pyr
100	94	ND	ND
120	108	0.03	0.03
140	123	0.18	0.18
160	147	0.52	0.52
180	168	1.48	1.48
200	187	2.18	2.18

[a]Average of duplicate determinations.
[b]Confirmed by mass spectrometry.

obtained from proline and N-Pro, respectively. Based on the N-Pro contents reported in Table 2 for the 150 ppm level of nitrite, this rate of conversion of N-Pro to N-Pyr would not be great enough to account for the levels of N-Pyr found in fried bacon (*3,6,11,23*). However, Nakamura et al. (*21*) reported that N-Pro is formed from proline and nitrite during the frying of bacon. Maximum N-Pro formation occurred at 125 C, above which temperature there was a gradual decrease in N-Pro content up to 175 C. At 200 C, there was a rapid decrease in N-Pro formation.

Gray et al. (*11*) reported an average value of 23.8 μM proline/100 g of uncooked bacon. This is equivalent to 27.4 ppm. From data in Table 3 it can be seen that a 0.33% conversion of proline to N-Pyr was obtained when the sample was heated in an oil bath at 200 C. Based on this percentage conversion, the free proline in bacon should theoretically produce approximately 90 ppb of N-Pyr during frying. When the conditions of 200 C (oil bath temperature) and 12 min were used in this study, the pork slices were overcooked. At an oil bath temperature of 180 C (maximum temperature of cooking system, 172 C), the pork slices were crisp but not burnt. Under these conditions, using the proline to N-Pyr factor of 0.21, bacon should theoretically produce 57 ppb of N-Pyr. This value is similar to those reported for fried bacon, assuming that during conventional frying procedures, approximately 35-40% of the N-Pyr is lost in the vapor (*10*).

Yields of N-Pyr obtained from N-Pro and proline in this study are in general agreement with the results of some of the previous studies. However, the absolute values obtained depend on the system used. Eisenbrand et al. (*4*) heated N-Pro in a sealed tube containing 2 ml of silicone oil and reported an 11% yield of N-Pyr at 230 C for 10 min. When ham containing added N-Pro was fried in a pan containing 3 ml of vegetable oil, yields of 0.24 and 0.42% N-Pyr were obtained when the samples were cooked at 180-190 C and 210-220 C for 8 min, respectively. These investigators explained these lower yields by reporting loss of N-Pyr in the vapor. The ham sample also was only heated to a temperature sufficiently high to effect decarboxylation on the outside whereas the inner part was obviously heated to a lesser extent. The water content of ham also contributes to lowering the effective cooking temperature.

In the present study, the temperature of the contents in the heating flask did not attain the same temperature of oil bath. The temperatures reported in Tables 3 and 4 were the maximum temperatures reached in the system after 12 min of heating. This temperature was only reached because some of the water in the pork slices was evaporated during the cooking process. For example, when the pork slices were heated in an oil bath at 180 C for 12 min, 25 ml of distillate was collected. When the system was cooked under reflux conditions at 180 C for 12 min, a maximum temperature of only 110 C was achieved. Since bacon is normally cooked in a frying pan with no lid attached, it is expected that higher temperatures are reached towards the end of the cooking process when the moisture content is decreased through evaporation.

Hwang and Rosen (*17*) heated a 250 mg slice (2-mm thick) of bacon to which N-Pro had been added at 185 C for 5 min and reported a 1.43% yield of N-Pyr. Under similar conditions, a yield of 0.33% N-Pyr was obtained from proline and nitrite. These bacon samples were cooked under reflux but because of the size of the bacon samples used, it would be expected that the moisture present did not greatly reduce the sample temperature below that of the oil bath.

This present study has shown that free proline in bacon can be N-nitrosated and decarboxylated to account for the presence of N-Pyr in fried bacon. This is consistent with the findings of Sen et al. (*24*) which support the pathway of free proline converted to N-Pro with the latter being decarboxylated during the frying process. However, as recently reported by Nakamura et al. (*21*), an alternative mechanism, decarboxylation of proline followed by reaction with nitrite is also a

possiblity. From these studies it was concluded that in the temperature range 100 to 150 C with a heating time of 10 min, the amounts of N-Pyr formed from free proline via pyrrolidine were almost the same as those formed via N-Pro. At temperatures of 175 C and above, the yield of N-Pyr via pyrrolidine formation was greater than that formed via N-Pro.

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