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STUDIES ON THE FLAVOR FRACTION OF BREAD CRUST ADSORBED BY CATION EXCHANGE RESIN¹

TERUHIKO MORIMOTO² AND JOHN A. JOHNSON³

ABSTRACT

A distinct bread-crust or soda-cracker aroma was produced by the reaction of proline and glucose. A similar aroma component was found in bread crust. The aroma components from both the reaction of proline and glucose and from bread crust were adsorbed on Amberlite IR-120 or CG-120 and eluted with 0.2N sodium hydroxide. These compounds showed a violet color reaction with ninhydrin, suggesting the presence of nitrogen. They were stable under acidic conditions against heating, and volatile under alkaline conditions. They showed an ultraviolet absorption maximum at 280 to 290 $m\mu$, with a slight difference between compounds from the proline-glucose reaction and those from the bread crust. By the use of gas chromatography, an aroma component was isolated from the reaction products of proline and glucose.

Several reviews of bread flavor have been published (1-5). Various investigations have related the presence of organic acids, esters, aldehydes, and alcohols to bread flavor. Most organic acids, esters, and alcohols are produced during liquid or dough fermentation (6-11). Aldehydes are produced during the fermentation process (7,11,13) as well as during the baking process, where the Maillard reaction occurs between amino groups and carbonyl compounds including reducing sugars (13,14). When amino acids participate in Maillard reaction, most frequently a carbonyl compound is produced with one less carbon atom than the amino acid from which it is formed. Furfural, hydroxymethylfurfural and pyruvic aldehyde are derived from reducing sugars (14,15).

However, the reaction of proline with glucose or dihydroxyacetone appears to be different. When proline is reacted with dihydroxyacetone,

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²Research Chemist, Tsukaguchi Division Laboratory of Confectionery Institute, Morinaga Seika Co., 400 Kamisakabe, Amagasaki City, Hyogo, Japan.

³Professor, Department of Flour and Feed Milling Industries, Kansas State University, Manhattan.

substances with a pleasant aroma are produced (16). Kiely *et al.* (17) described the flavor produced from the reaction of proline and glucose as cornlike. Wick *et al.* (18) reported that bread made with added proline was preferred on the basis of its aroma.

The present investigation was made to examine the properties of the substances produced in soda crackers or bread crust by the reaction of proline with glucose. Attempts were made to isolate and characterize these substances.

Materials and Methods

Preparation of Bread Crust. Bread crust was produced by baking a thinly sheeted dough produced by a liquid pre-ferment process. The liquid pre-ferment was made as follows:

Water	400 ml.
Dextrose	15 g.
Compressed yeast	25 g.
Yeast food	7.5 g.
Sodium chloride	15 g.
Nonfat dry milk	15 g.
Monopotassium hydrogen phosphate	1.2 g.
Disodium hydrogen phosphate	0.7 g.

The pre-ferment was held for 3 hr. at 30°C. before the addition of flour (700 g.) and dextrose (50 g.). The dough was mixed to optimum development, and fermented for 45 min. at 30°C., and divided. After a 5-min. rest period, the doughs were sheeted to a thickness of approximately $\frac{1}{4}$ in. The doughs, placed in large sheet pans, were proofed for 30 min. at 35°C. After proofing, the doughs were baked at 230°C. until a desired crust color was obtained. Shortening was eliminated from the formula to assist in extraction of the compounds from the crust and eventual separation by a cation-exchange technique.

Reaction of Amino Acids and Glucose. Individual amino acids (0.01 mole) were reacted with glucose (0.01 mole) by heating in an autoclave at 120°C. for 30 min. The test tubes containing the reactants in a phosphate buffer (0.025M, pH 7) were capped with cotton during the heating. Aroma of each reaction mixture was observed after cooling. The products of proline-glucose reaction had a distinct cracker aroma and were used for further isolation studies. Preliminary experiments indicated that similar aromas were produced by heating at a pH of 4.5 but that the rate of formation of the aromas was greater under neutral or alkaline conditions.

Isolation of the Aroma Fraction of Bread Crust and of Proline-Glucose Reaction Mixture. Small pieces of bread crust were refluxed at 65°C. with 70% ethyl alcohol for 1 hr. The extraction process was

repeated three times and the extracts were combined and filtered. The filtrate was concentrated at 65°C. under vacuum. The concentrate was adjusted to pH 2.5 and placed on a column of Amberlite IR-120 (20- to 50-mesh) which had been buffered at pH 5.28 with 0.35N citrate buffer. The column was washed with a citrate buffer (pH 5.28) to remove free amino acids and peptides (19) until the effluent gave a negative reaction to ninhydrin. The fraction having a distinct aroma was then eluted with 0.2N sodium hydroxide. Elution of the aroma fraction was detected by color, aroma, and reaction with ninhydrin.

In another experiment, in order to prepare larger quantities of the aroma fraction from the proline-glucose reaction, 0.1 mole of proline and glucose in 30 ml. of a 0.025M phosphate buffer (pH 7) were reacted under reflux at 100°C. for 1 hr. At the end of this time the mixture (after cooling) was acidified to pH 2.5 with hydrochloric acid. This reaction mixture was placed on the Amberlite CG-120 (200-mesh) which had been buffered at pH 3.25 with a citrate buffer (0.2N). The column was washed with the citrate buffer (pH 3.25) to remove unreacted proline (19). The aroma fraction was eluted with 0.2N sodium hydroxide.

Paper Chromatography. The aroma substances isolated by the use of the Amberlite resin column were further purified by paper chromatography. For this purpose, Whatman No. 1 filter paper was developed for 5 hr. with a butanol-acetic acid-0.1N hydrochloric acid solution (3:1:2 v./v.) at 25°C. Larger quantities of the aroma substance were prepared by stripping a filter paper with the column eluate and developing in the normal manner. A section of this paper was sprayed independently with ninhydrin to identify the aroma substances. A broad band stained violet; a second band stained yellow. The second paper section was then cut according to the identification and the compounds were eluted from the paper with 0.02N hydrochloric acid or water. Ultraviolet absorption spectra of the fractions were measured with a Beckman Model DU spectrophotometer.

Gas Chromatography. An Aerograph gas chromatograph (model A-90-p) with hydrogen-flame ionization detector (model 500) was used to investigate the aroma components. A 1/8-in., 10-ft. stainless-steel column packed with Chromosorb W (60- to 80-mesh), coated with 10% Carbowax 20M and 4% potassium hydroxide was used. Helium was used as the carrier gas.

Results and Discussion

Reaction between Various Amino Acids and Glucose. The results, summarized in Table I, indicate that the amino acids reacting with

TABLE I
AROMA PRODUCED BY HEATING AQUEOUS AMINO ACID-GLUCOSE MIXTURE

AMINO ACID	DESCRIPTION OF AROMA	AMINO ACID	DESCRIPTION OF AROMA
Serine	Moderate	Histidine	Moderate
Glutamic acid	Moderate	Proline	Strong; breadcrust, cracker
Hydroxyproline	Weak	Threonine	Weak
Aspartic acid	Strong; breadcrust	Cystine	Strong; hydrogen sulfide
Tyrosine	Weak	Methionine	Strong; baked potato
Alanine	Moderate; breadcrust	Glycine	Moderate
Arginine	Weak	Phenylalanine	Strong; flower
Isoleucine	Moderate; breadcrust	Lysine	Weak
Valine	Moderate; breadcrust	Leucine	Moderate; breadcrust
Tryptophan	Strong		

glucose gave various characteristic aromas. The aroma of certain amino acids could not be expressed. Aspartic acid, alanine, leucine, isoleucine, valine, and proline developed characteristic aromas reminiscent of bread crust flavor, although they were not all of the same character. Particularly, proline gave a crackerlike aroma that was different from that of certain carbonyl compounds. Compared with the results of Kiely *et al.* (17), the aroma of leucine agreed with their finding, but arginine and histidine did not give a significant aroma under the present experimental conditions. Kiely *et al.* (17) described the aroma of the proline reaction as being cornlike. In the present investigation, the aroma from the proline-glucose reaction appeared sometimes to be cornlike, particularly after the completion of the reaction; but, after storage for a short time, the odor was distinctly like that of bread crust or soda cracker. It was assumed that the aroma changed with storage and subsequent volatilization of carbonyl compounds present. In regard to the cracker aroma, Wiseblatt and Zoumut (16) reported similar results from the reaction of proline with dihydroxyacetone. It is now apparent that proline not only reacts with dihydroxyacetone to produce aromatic substances but will readily react with glucose which is usually present in abundant amounts in a dough to produce the crackerlike aroma. Under the conditions of bread-crust formation, the temperature is elevated and the pH is moderately low. These conditions favor the formation of the aroma substances resulting from the reaction of proline and glucose.

Sometimes valine also produced a similar crackerlike aroma. Pyruvic aldehyde or ascorbic acid with proline also produced a similar, but weak, flavor. These observations suggested that the aroma substances might be produced from the proline structure rather than from the glucose via pyrrolidine as suggested by Wiseblatt and Zoumut (16).

Column Chromatography of the Proline Reaction Products. The

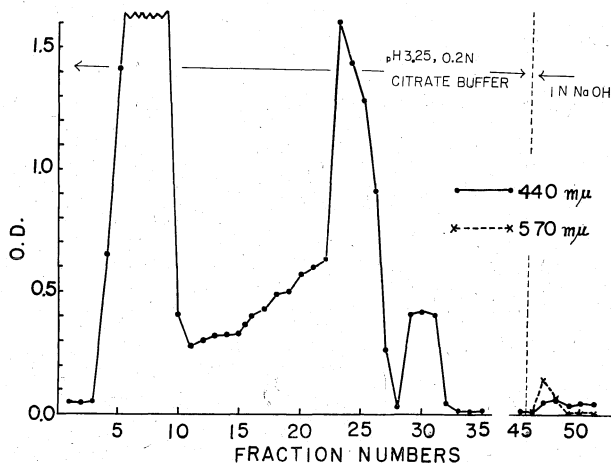


Fig. 1. Elution pattern of the reaction mixture between proline and glucose on Amberlite CG-120. Each fraction was 4.5 ml.

reaction products of proline and glucose were adsorbed and separated on an Amberlite CG-120 column (1.3×22 cm.) as shown in Fig. 1. The several peaks resulting from elution with a buffer at pH 3.25 contained brown pigments and reacted with ninhydrin to give a reddish-brown color. Each fraction was reacted with ninhydrin and expressed as absorbances (O.D.) at $440 \text{ m}\mu$. The first material eluted contained carbonyl compounds and brown pigments. The presence of carbonyl compounds was established by formation of 2,4-dinitrophenylhydrazone derivatives. No detailed study was made of these substances. The second peak contained unreacted proline and the third peak was unknown. Beginning with the 45 fraction, a substance was eluted from the column which had properties different from those eluted previously. This substance was eluted with 1N sodium hydroxide and exhibited a violet color in contrast with the brown color of previous fractions when reacted with ninhydrin. A small amount of brown pigment also was eluted at the same time with the flavor compounds. Each fraction was reacted with ninhydrin and expressed as absorbance at $570 \text{ m}\mu$ as well as $440 \text{ m}\mu$. Because of the violet color reaction with ninhydrin, the presence of nitrogen in this compound was suggested. The 47 and 48 fractions had distinctly the aroma of bread crust or soda cracker. In the present paper, the cracker aroma should be defined as part of the bread-crust aroma.

The elution pattern from the Amberlite CG-120 column of the compounds from the dihydroxyacetone and proline reaction are shown in Fig. 2. In this case, fractions eluted with citrate buffer (pH 3.25) had

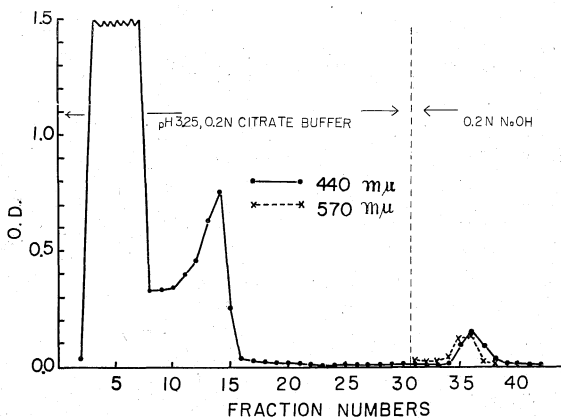


Fig. 2. Elution pattern of the reaction mixture between proline and dihydroxyacetone on Amberlite CG-120. Each fraction eluted with the pH 3.25 buffer was 8.5 ml.; the one eluted with 0.2N sodium hydroxide was 4.5 ml.

a volume of 8.5 ml. The first large fraction contained brown pigments and unknown carbonyl compounds. The second peak contained unreacted proline, and the third fraction, eluted by 0.2N sodium hydroxide, contained a substance with a cracker aroma in addition to pigments. This aromatic fraction also reacted with ninhydrin to give a violet color which exhibited stronger light absorption at 570 $m\mu$ contrasted with the substances eluted previously with the pH 3.25 buffer.

Although the amount of the aromatic substances present in the reaction of proline with dihydroxyacetone or glucose was less than the amount of pigments and carbonyl compounds, the aroma was distinct and it was the same for both the reactions with glucose and dihydroxyacetone.

Preparative Column Chromatography of Bread-Crust Flavor. In order to prepare a larger quantity of flavor substances associated with bread crust, another column consisting of Amberlite IR-120 (20- to 50-mesh) was prepared. This column (2.3 \times 17.0 cm.) was buffered with 0.35N citrate buffer at pH 5.28. Approximately 1,000 g. of bread crust was extracted with 70% ethyl alcohol, and 100 ml. of the concentrate was adjusted to pH 3.0 and placed on the column. All the free amino acids present in the cloudy bread-crust extract were eluted from the resin with the citrate buffer at pH 5.28. The citrate buffer was followed by elution of the proline-glucose reaction products with 0.2N sodium hydroxide. The fraction containing brown pigments and the aroma compounds were collected, neutralized with hydrochloric acid, and concentrated under vacuum. The concentrate appeared to have an

aroma similar to that of the proline-glucose product but less distinct.

A larger quantity of the products of the proline-glucose synthetic reaction was prepared as described in "Materials and Methods." Amberlite CG-120 was used instead of IR-120 in this experiment. Both the flavor components from bread crust and the proline-glucose reaction had less aroma under acidic conditions (about pH 2). When such an acidic sample was placed in the mouth, the flavor was somewhat reminiscent of the soda-cracker flavor. When the compounds isolated by the columns were kept under alkaline conditions, the flavor intensity diminished, suggesting that they are unstable or volatile under such conditions. It is speculated that as pH of bread is usually acidic, such a flavor could be kept without vaporizing even when other kinds of flavor substances like aldehydes, alcohols, and organic acids would be vaporized during and after baking.

Paper Chromatography of Aroma Compounds. The concentrated aroma fraction from the glucose-proline reaction isolated by column chromatography showed a different R_f value from that of glucosamine, 2-pyrrolidone, pyrrolidine, butanol amine, isobutylamine, n-ethylamine, n-propylamine, n-amylamine, or n-butylamine.

The aroma fractions obtained from the bread crust and the proline-glucose reaction were compared by paper chromatography. An example of the results is shown in Fig. 3. Separation by paper chromatography

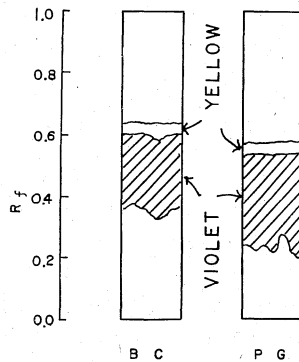


Fig. 3. Paper chromatogram of flavor fractions of bread crust and proline-glucose reaction: BC, bread crust; PG, proline-glucose product.

indicates that two substances were present. One produced a large spot of the violet color and the second, a small spot of a yellow color when sprayed with ninhydrin. Water extracts of the two fractions on another paper corresponding to the violet and the yellow part were investigated by ultraviolet absorption. These results are shown in Fig. 4. When

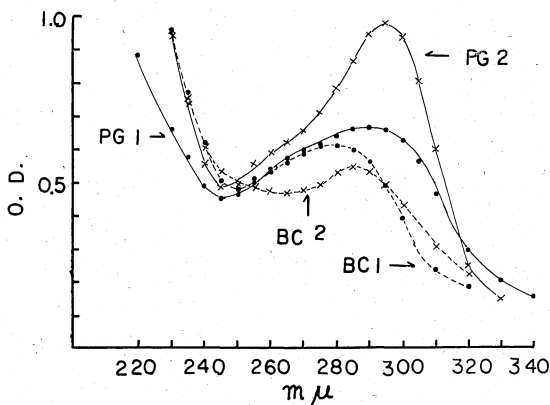


Fig. 4. Ultraviolet absorption spectrum of flavor components extracted from the paper. Curves BC 1, PG 1, and BC 2, PG 2 correspond to the violet and yellow areas in Fig. 3, respectively.

the water extracts from the paper chromatogram were concentrated and tested organoleptically, the violet color fraction of BC or PG in Fig. 3 exhibited the aroma. The aroma was distinct in the case of proline-glucose, but weak in the case of the bread crust extract. The yellow color fraction did not exhibit the aroma in both cases. The aroma fraction, PG 1 in Fig. 4, had an ultraviolet absorption pattern similar to that reported by Wiseblatt and Zoumut (16); the spectrum of BC 1 fraction from bread crust was slightly different. This result suggests that the aroma compound of bread crust may be different from that of proline-glucose products.

Gas Chromatography of Aroma Compounds. The concentrated aroma fraction obtained by preparative chromatography was made alkaline with sodium hydroxide and subjected to gas chromatography. As shown in Fig. 5, the aroma fraction of the proline-glucose reaction showed two main peaks, A and B. Some peaks of impurities from sodium hydroxide appeared before A and B. A small peak having the same retention time as peak A appeared in the sodium hydroxide blank. Therefore it is not certain that peak A represents one of the flavor components; but it was certain that peak B represented a component of the aroma compounds. Peak B was always obtained in the gas-chromatographic tests and was reproduced in the experiments with head-space gas analysis. In a smelling test of peak B from the gas chromatogram, the cracker aroma was indicated.

The retention time of peak B was somewhat different from that of pyrrolidine, which is a possible intermediate substance of this component (16). The chromatogram of pyrrolidine is shown in Fig. 6.

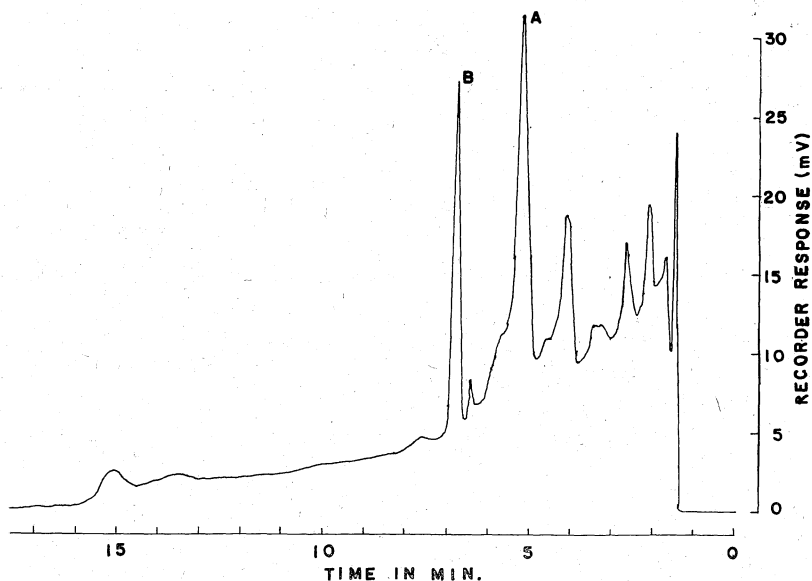


Fig. 5. Gas chromatogram of flavor components from the proline-glucose products. Conditions: Carbowax 20M-KOH column; helium, 17.6 ml. per min.; column temp., 120°C.; injector temp., 180°C.; and 5 μ l. of the aqueous sample.

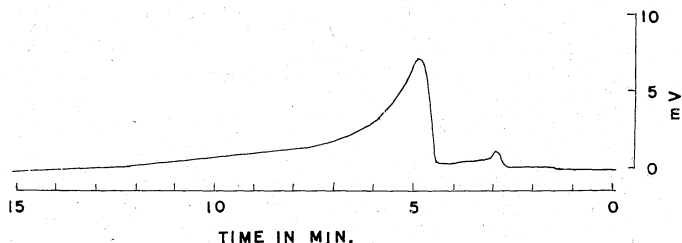


Fig. 6. Gas chromatogram of pyrrolidine on the Carbowax 20M-KOH column. The conditions were the same as in Fig. 5. Forty μ l. of head-space gas of pyrrolidine was injected.

The odor component of proline degraded by periodate was identified as 1-pyrroline by Yoshikawa *et al.* (20). The retention time of 1-pyrroline prepared in the present investigation by the ornithine-ninhydrin (21) or the proline-sodium metaperiodate reaction (22) was different from that of the peak B substance. Kobayashi and Fujimaki (23) reported that n-acetyl pyrroline was formed with the reaction of glucose and proline under dry heat rather than in a buffer solution. There is a possibility that the aroma compound (peak B) in the present work may be the same compound.

With bread crust, gas chromatography failed to reveal a significant

peak corresponding to peak B. There may be two reasons for this. The amount of aroma compounds was too small to be estimated, or the aroma component was different from that from the proline-glucose reaction, in spite of similarities observed by paper and resin column chromatography of the aroma.

Wick *et al.* (18), in spite of obtaining a preferred organoleptic flavor by adding proline to bread dough, could not demonstrate a difference by gas-chromatographic analysis. There is a possibility that the column used in their work was not appropriate for the compounds resulting from the proline reaction. The peak, like B in Fig. 5, could not be obtained by the use of a Quadrol-packed column, while the Carbowax 20M column treated with potassium hydroxide was useful in detecting the peak due to the aroma component from proline.

Further study should be done on the identification of this component.

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