EFFECT OF PROTEASE ENZYMES ON BREAD FLAVOR

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Egypt, U. A. R., 1961

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Flour
and Feed Milling Industries

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1966

Approved by:

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TO

THE PEOPLE OF

UNITED ARAB REPUBLIC

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INTRODUCTION AND REVIEW OF LITERATURE

Interest in the flavor of bread probably is as old as the art of baking. Certainly, this interest has grown in recent times because so much ingenuity has gone into developing a science of baking and because the products of bakers face such severe competition for the consumer's food budget.

The delectable flavor of freshly baked bread deteriorates rapidly after a relatively short storage period, resulting in a much less appealing product. Also, it is generally believed that modern bread production methods produce less flavorful bread. Moreover, the modern trend toward mechanization and wider distribution of bread increases the magnitude of this problem. A better product would be available to consumers if methods of enhancing and stabilizing bread flavor could be developed. This should also tend to increase the per capita consumption of bread.

The use of specific enzyme systems having desired properties offers many possibilities for control in the production of bread. Interest in the use of proteases in breadmaking was stimulated greatly by the introduction of fungal enzyme concentrates prepared from cultures of Aspergillus Oryzae in 1950. In addition to the desirable effects of fungal amylases and proteases on dough properties, the effect of these enzymes on the properties of bread may also be desirable.

The primary objective of the present work was to study the effect of using different concentrations of Rhozyme J-25 and papain enzymes on bread flavor and to provide data on production

of certain carbonyl compounds in white bread crust as affected by using these enzyme systems.

Flavor and Organoleptic Techniques. One of the serious problems is that bread flavor can not be objectively defined. Aroma is described as a distinctive characteristic suggestive of fragrance or odor (11). Odor is a sensation due to stimulation of the olfactory receptors in the nasal passages by gaseous material and pure odor or true odors are olfactory experiences unaffected by the action of other senses of the skin and mouth, especially the trigeminal and taste receptors. Taste, by contrast, is another of the senses, the receptors for which are located in the mouth and are activated by a large variety of different compounds in solution. Most investigators usually limit gustatory qualities of foods to four factors: saline, sweet, sour and bitter. The flavor of a substance is a mingled unitary experience which includes sensations of taste, smell and pressure and often other cutaneous sensations such as warmth, cold or mild pain. These are attributes of foods, beverages and seasonings resulting from the stimulation of the senses which are grouped together at the entrance to the alimentary and respiratory tracts (11). Therefore, "good taste" of a food product includes the total sensations experienced by the consumer, such as odor, taste, warmth, freshness, appearance and mechanical eating qualities.

Research on bread flavor may take the route of consumer preference tastes which are defined as expression of higher degree of liking or choice of one object relative to others, including

psychological continuum of affectivity (pleasantness - unpleasantness) on which such choices are based. This continuum also is referred to as that of degree of liking or disliking. The flavor profile technique is a method of qualitative description analysis of aroma and flavor. This method makes it possible to indicate degrees of difference between two samples on the basis of individual character notes and the degree of blending and the over-all impression of the product. Difference tests are a comparison or a test of quality variation without indication of preference. This method can be applied in two ways: Dual standard method of difference testing may be used in which two samples are identified and presented to the judges first as knowns and later as unknowns, or Duo-trio method of difference testing in which one of a pair of samples is identified and presented first, then the observer receives two more samples as unknowns in random order. The time interval can be varied as desired. The observer's task is to pick the sample that is different. A combination of these organoleptic procedures may be used.

Taste panels were employed by Cathcart (9), Ingels et al. (18) and King et al. (29) to determine bread flavor characteristics affected by various physical factors occurring in the breadmaking process. The formula, method of mixing, flour grade, class of wheat, fermentation time and temperature, proof time and temperature, and freshness of the bread were some of the factors studied. Though such tests indicate consumer preferences, they contribute little to the basic knowledge of the specific chemical stimuli involved. Ingels (18) found that the best procedure was

to use sliced bread, wrapping two slices together in a 10" x 12" piece of water-proofed cellophane paper held together by a rubber band. According to Platt (11), three samples of bread is about the maximum number that could be judged with any degree of accuracy at one time by a judge. They concluded that freshness affects flavor more favorably than any other single characteristic of bread. The judging of odor and taste in bread is not a simple procedure and greatest care is necessary in experimental design. King et al. (29) stated that the use of a large number of judges does not increase the validity of the results of the test on bread flavor. A large untrained group of judges are no more accurate than a small group of trained judges. Ingels (18) stated that six judges gave adequate coverage; therefore, there may be any multiple of six judges, and 36 judges are very convenient. The Committee on Sensory Evaluation of the Institute of Food Technologists (11) recommend the number of 3-10 trained judges, 8-25 semi-trained judges and over 80 untrained judges for rank order of type test when number of samples per test are 2-7.

Cathcart (9) pointed out that factors other than ingredients influence bread flavor. Thomas and Rothe (71) stated that the presence of a substance in concentrations above the threshold of human perception must be established before it may be assumed to be a component of bread flavor. Also, interaction between compounds may alter the threshold level. Wiseblatt (76) pointed out that the non-additive nature of mixed aromas and the vapor pressure of the compounds arising from bread are important to flavor. Baker and Mize (4) found that, in addition to the ingredients,

bread flavor depends both on the fermentation products and on compounds formed in the crust during oven baking. Johnson (20) stated that the detection of an aromatic substance in bread does not necessarily mean it is involved in bread flavor, and research combining chemical analysis with consumer preference studies is sorely needed.

Bread Flavor Stimuli. Nearly 70 identified compounds which are thought to contribute to bread flavor have been reported either in pre-ferment, dough, oven vapors or bread. Number of unidentified compounds also have been observed (9, 11). Pence (51) found that less than 1 percent of the oven vapor condensate consisted of organic compounds which were classified as acids, alcohols, esters and carbonyl compounds. Hunter et al. (16) obtained 44 different peaks on gas chromatograms from the flash exchange of ethyl esters of organic acid concentrates of pre-ferment. Only 28 peaks, all mono-acids, were identified by comparison of retention times. These acids were formic, acetic, propionic, iso-butyric, n-butyric, n-valeric, iso-valeric, crotonic, iso-caproic, caproic, heptylic, caprylic, pelargonic, capric, lauric, myristic and palmitic. In general, organic acids have been extracted from pre-ferment, dough and bread (7, 25). Ronnebeck (56) and Thomas and Ronnebeck (68) determined volatile and non-volatile acid content of rye bread but could not establish positive correlation between acidity and organoleptic tests. The significance of organic acids in bread flavor is not known. Johnson (20) postulated that their effect was a subtle working through their effect on the physical properties of the crumb and

that they made no direct contribution to flavor. Hunter et al. (16) thought that the higher acids might also function to influence bread flavor by hindering the evaporation of lower boiling components.

Smith and Coffman (67) found aldehydes, ketones, esters, alcohols and diols among 27 different neutral components in the ether extracts of a centrifuged pre-ferment. The steam distillate contained propyl, iso-butyl and iso-amyl alcohols in addition to a large quantity of ethyl alcohol. In the non-volatile portion of the pre-ferment, they found alpha-butyrolactone, 1,3-propanediol, 2-phenylethanol and levo and meso 2,3 butanediol. They (67) believed that lower alcohols from pre-ferment were not involved in flavor, but the higher alcohols present only in trace amounts tended to remain during baking as flavor constituents in bread. Smith and Coffman (67) isolated ethyl formate, ethyl acetate and 1,3-propanediolmonoacetate from pre-ferment as esters components. Wiseblatt (78) isolated ethyl pyruvate, ethyl levulinate, ethyl succinate, ethyl hydrocinnonate, ethyl benzoate, ethyl acetate and ethyl itaconate from bread. Johnson (20) reported that higher esters tend to remain but they were not formed in large quantities because the reactants, alcohol and organic acids were present only in low concentrations.

Many carbonyl compounds have been isolated from pre-ferment, dough and bread. Wiseblatt and Kohn (77) recovered the volatiles from shredded bread by dry distillation under very high vacuum, thereby hoping to minimize alterations to unstable constituents. They were able to identify acetaldehyde, acetone, crotonaldehyde,

diacetyl, furfural, 2-ethylhexanal, 2-hexanone, 3-heptanone and pyruvaldehyde. Ng et al. (49), in addition to the above-mentioned carbonyl compounds, isolated and identified formaldehyde, methylethylketone, 2-methylbutyraldehyde, n-hexanal and ethyl pyruvate in fresh, white bread. Iso-butyraldehyde and n-valeraldehyde were detected in oven vapors only. Miller et al. (47) removed the volatile constituents formed during fermentation with a stream of inert gas and converted the carbonly compounds to 2,4-dinitrophenylhydrazone derivatives. They were able to isolate and identify formaldehyde, acetaldehyde, acetone, butyraldehyde, isobutyraldehyde, methylethylketone, 2-methylbutanal, n-valeraldehyde by paper, column and gas chromatography. Linko et al. (37) performed quantitative analyses of carbonyl compounds in pre-ferments and found that acetaldehyde was the major component. Other carbonyl compounds included acetone, propionaldehyde, formaldehyde, iso-butyraldehyde, methylethylketone, n-valeraldehyde, iso-valeraldehyde, 2-methyl butanal and n-hexanal.

Linko et al. (35) showed no effect of different methods of bread production on the amount of certain carbonyl compounds found in crust and crumb. They also found the carbonyl content in the crust of wrapped or unwrapped bread decreased as bread aged. Thomas and Rothe (70), relating the flavor of different types of bread, found the aldehyde content was influenced by baking time and temperature. They showed that the aldehydes migrated from the crust into the crumb during storage. Quantitative values for acetaldehyde, furfural, iso-valeraldehyde, pyruvaldehyde, acetone, acetoin, iso-aldehyde and diacetyl were

given by Rothe and Thomas (61) for crust and crumb of white, brown rye and whole meal rye breads. The aldehyde content increased with increasing darkness of bread, roughly corresponding to organoleptic flavor intensity.

Barnes and Kaufman (5) observed that a flavor similar to that of fresh bread could be obtained by reacting leucine with a reducing carbohydrate. Kretovich and Tokareva (32) and Lüers (39) reported that reactions between various amino acids and sugars, eventually leading to the formation of brown pigments, were accompanied by the formation of furfural and other volatile aldehydes which were responsible for the aroma. Kiely et al. (28) reacted individual amino acids with various reducing sugars under a range of temperature, moisture contents and pH values evaluating the aromas produced by a flavor profile technique. As a result, they found that leucine, when added to an instant bread mix, enhanced the aroma of the finished loaf of bread. According to Rothe and Thomas (61), volatile aldehydes formed during non-enzymatic browning reactions are a major factor in bread flavor. They observed a close relationship between crust browning and the quantity of both total aldehydes and furfural. Furfural appears to be the most important aldehyde in rye breads. The total aldehyde content of bread is influenced markedly by the baking time. Slow-baked products, such as pumpernickel, have higher aldehyde content than various rapidly baked varieties. Also, they reported that the most reactive amino acids were, in the order of decreasing reactivity, iso-leucine, leucine, valine and methionine.

The formation of compounds responsible for bread flavor has been believed to be closely related to the Maillard-type browning during oven baking (17). Kretovich and Tokareva (32) related bread flavor with the presence of furfural and hydroxymethylfurfural. Simultaneously, furfural was formed from pentoses and hydroxymethylfurfural from hexoses, known intermediates of nonenzymatic browning. They also observed that bread flavor was related to the total aldehyde content. Komm and Lehman (30) related bread flavor to the presence of furfural and hydroxymethylfurfural. Miller et al. (47) found that furfural was absent from pre-ferments and concluded that it was formed during baking. Linko et al. (36) considered hydroxymethylfurfural (HMF) to be formed readily from hexoses at baking temperature. Increasing the initial sucrose concentration in the formula increased the production of HMF in the crust. The use of fructose, in particular, increased the HMF concentration. Furfural, as well as arising from pentoses, could also arise in small amounts from hexoses during baking. Rotsch (58) found that the furfural content rose with the amount of sugar and protein present in various baked goods. Furfural also was higher in well-baked bread. Although the presence of furfural was believed to be associated with the browning reaction, Rotsch and Dorner (59) found it present in low concentration in bread baked without crust formation. It has been suggested that insoluble proteins were more important than free amino acids as browning reactants in bread baking (15). When dextrose, egg white and gluten were added, a definite improvement in crust color was observed (15).

Thomas and Rothe (67) and Linko et al. (36) found the concentration of carbonyl compounds in crust far exceeded that in crumb, indicating the important role of oven browning in formation of carbonyl compounds. Schoch (65) proposed that some of the aldehydes may complex with the amylose starch present in the crumb. He postulated, upon re-heating, that the helical structure was disrupted and the carbonyl compounds were released which might account, in part, for the refreshening of stale bread by heating. Refreshening of bread by heating may be due also to production of additional compounds by the browning reaction, in addition to physical changes which occur. Rooney (57) concluded that dipeptides produced color and carbonyl compound during baking. Wheat gliaden and n-hexanoic acid also caused small increases in color intensity and significant increases in carbonyl compounds. Also, the aroma of bread can be altered by addition of amino acids and peptides to the formula.

Salem (64) indicated that volatile aldehydes produced by the Strecker degradation, by which an amino acid gives rise to an aldehyde with one less carbon atom, are regarded as the most important products of the reaction. The large quantities of carbonyl compounds in the crust suggest that the reaction occurs mainly during crust formation. The fact that no corresponding increase was observed in the aldehyde content of the crumb when amino acid was added, indicated that the carbonyl compound content present in the crumb is composed largely of aldehydes produced during fermentation. Therefore, browning reactions may contribute only slightly to the total carbonyl content in the crumb.

The significance of carbonyl compounds in bread flavor has been demonstrated by the positive correlation of the organoleptic tests and total aldehyde content by various investigators (8, 71).

Protease Systems in Bread Production. Enzymes are classified according to the substrate on which they act. In cereal enzymology, major attention has been devoted to the amylases and proteases. Less attention has been given to the lipases, phosphatases, phosphylases, oxidases, dehydrogenases and decarboxylases, although they, too, are important (21).

Protease systems are of great interest to cereal chemists from both practical and theoretical standpoints. There is little knowledge regarding the specificity of the protease enzymes found in sound or germinated cereals. It is likely that the proteases extractable from flour or from malted flour represent a series of enzymes. Some have exopeptidase activity and others have endopeptidase activity. McConnell (42) found that exopeptidase activity in malted wheat catalyzed the splitting of internal protein bonds of edestin, gelatin and wheat gluten. Dipeptidases that accelerate the splitting of leucylglycine and glycylglycine also were found (21). Johnson and Miller (22) have reported that concentrated active enzymes from microorganisms are utilized by the baking industries and the use of specific enzyme systems having desired properties offers many possibilities for control in the production of bread. However, additional fundamental information is required to complete the picture. Miller and Johnson (46) concluded that enzymes can be used to modify dough properties as well as to improve the flavor imparting quality of

the ferment. Also, the use of fungal protease preparations is desirable. In addition to the desirable effects of fungal amylase and protease on dough properties, the effects of these enzymes on the properties of the bread also are desirable. Supplementing flour with both alpha-amylase and proteases from fungal origin may produce loaves that are symmetrical with soft grain and texture characteristics.

Johnson et al. (27) have reported that different peptide bonds of gluten were split by the various enzyme systems. The pH optimum is influenced by the nature and concentration of the substrate. Inactivation temperature of proteinase probably is close to 55-60°C. at the hydrogen ion activity of fermenting dough, according to Miller and Johnson (45). They also postulated that proteinase is active during the baking process for only a short time. Therefore, the action of the proteinases may be expected to occur during the long period of sponge fermentation, particularly since after addition of salt to the bread dough, approximately 60 percent of the proteinase activity is inhibited (44). The activity of the proteinase during the proofing and baking processes would appear to be rather limited and of minor significance. Miller and Johnson (46) indicated that the action of amylases during sponge fermentation is limited by the starch availability. Moreover, the dough consistency decreased proportionally to the increased concentration of protease. Excessive protease action can cause stickiness in dough, however, and will cause undesirable results. For this reason, Miller and Johnson (46) indicated care should be exercised in determining the optimum

level of protease. Also, various flours required different quantities of alpha-amylase and protease. Normal flours tolerated a range of enzyme activity within which satisfactory bread could be produced. It also became clear that the upper level, particularly of fungal amylase, was not critical. The optimum level for protease supplementation was within a somewhat narrower range than that for amylase. Miller and Johnson (46) revealed that the requirements of different flours for enzyme supplementation are not related to the flour source, type of milling, protein content or the Amylograph viscosity of the unsupplemented flour. The requirement for protease supplementation, in particular, can be determined only by the baking test. Pomeranz et al.(54) have demonstrated that the effects of proteases evaluated by the rheological test are in the same order as determined by actual breadmaking.

The present work was undertaken to study effect of using different concentrations of Rhozyme J-25 and papain enzyme preparations on production of carbonyl compound in white bread crust, on bread flavor and on bread properties.

MATERIALS AND METHODS

Determination of Proteolytic Activity. The modified Ayre-Anderson procedure (2) was used to determine the proteolytic activity of Rhozyme J-25 and papain enzyme preparations.

Preparation of Enzyme Solutions. Enzyme solutions were prepared by extracting the dry enzyme preparation with acetate

buffer solution at pH 4.10 ± 0.05 (glass electrode pH meter) at 40°C.

Digestion Procedure. Two and one-half grams of Difco Bacto-Hemoglobin[1] on a dry basis and approximately 2 grams of washed finely powdered pumice were placed into two 125-ml Erlenmeyer flasks. A volume of 48 ml of acetate buffer solution, pH 4.7 at 40°C., was added with 2 ml of the appropriate dilution of the proteolytic enzyme solution. The flasks were tightly stoppered and placed in an automatic shaking device fitted in a constant temperature bath held at 40°C. ± 0.1°C. Ten ml of trichloro-acetic acid solution (180 grams in 320 ml water) were added to the flask at the end of 15 minutes of digestion and to the second flask after 5.25 hours of digestion. Each flask was shaken and kept in the water bath at 40°C. for exactly 30 minutes. The suspension was filtered and a 10-ml aliquot was pipetted directly into Kjeldahl flasks and soluble nitrogen determined by using the Kjeldahl nitrogen method (3).

Expression of Proteolytic Activity. Proteolytic activity was measured by differences in titration volumes of 15 minutes and 5.25 hours of digestion expressed as ml 0.0714 N NaOH (mg increase in soluble nitrogen). This value was transformed to mg increase in soluble N (N) per 10-ml aliquots multiplied by 3/2 power. This value was multiplied by 6 (total final volume of digest divided by 10-ml aliquot) and by 1000/mg enzyme source.

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[1] Difco Laboratories, Detroit, Michigan.

This value was the activity expressed in hemoglobin units per gram.

$$\text{H.U. per gram} = \frac{N^{3/2} \times 6 \times 1000}{\text{mg enzyme}}$$

Enzyme Preparation. The commercial proteases Rhozyme J-25* and papain**, were used as enzyme sources. Rhozyme J-25 enzyme preparation was produced from Aspergillus Oryza and papain was of plant origin.

A summary of protease activity, as determined by the Ayre-Anderson procedure, expressed in hemoglobin units per gram of enzyme preparation, is given in Table 1.

Table 1. Summary of protease activity of papain and Rhozyme J-25 enzyme preparations.

Enzyme preparation	Protease activity as hemoglobin units per gram
Papain	1,412
Rhozyme J-25***	18,523

* Rohm and Haas Company, Washington Square, Philadelphia, Pa.

** Nutritional Biochemical Corporation, 21010 Miles Ave., Cleveland, Ohio.

*** Rohm and Haas Company Bulletin, December 1964, reported that Rhozyme J-25 enzyme preparation contained 8000 S.K.B. units of alpha-amylase per gram.

Enzyme Concentrations. Different amounts of each enzyme preparation were used to give seven uniform levels of hemoblogin units per 700 grams of flour. The following table indicates the amount in mg of Rhozyme J-25 and papain enzyme preparations per 700 grams of flour and the corresponding H.U. activity levels.

Table 2. Enzyme weights having equivalent activity.

Enzyme activity H.U./700 grams flour	Mg enzyme preparation/700 grams flour	
	Papain	Rhozyme J-25
0	0.00	0.00
75	53.20	4.05
150	106.35	8.10
300	212.65	16.20
500	354.20	27.00
700	495.80	37.80
1000	708.30	54.00

Preparation of Enzyme Extracts. Enzyme solutions were prepared by extracting the dry preparations for 30 minutes at 30°C. with distilled water immediately prior to use. The enzyme extract was added with water during mixing of the sponge dough.

Bread Baking. One-pound loaves of bread were baked on a laboratory scale, employing the sponge dough procedure. The formula used for baking one-pound loaves is given in Table 3.

Table 3. The bread formula.

Ingredients	Composition	Sponge	Dough
	%	grams	
Flour (14% protein)	100	490	210
Arkady	0.5	3.5	--
Yeast	3	21	--
Water	65	318	137
Sugar	4	--	28
Sodium chloride	2	--	14
Shortening	3	--	21

The sponge was mixed for 2 minutes, fermented at 86°F. and 90 percent relative humidity. After 4 hours fermentation, the dough was remixed with the rest of the ingredients to optimum

consistency, followed by 30 minutes fermentation and allowed 10 minutes rest before molding. Proofing time was 50 minutes (98°F., 95% R.H.). The bread was baked 28 minutes at 430°F.

Determination of Water-soluble Nitrogen in the Dough after Fermentation. The three different enzyme concentrations, zero H.U. (as control), 300 H.U., 500 H.U. and 1000 H.U. per 700 grams of flour, were used for both papain and Rhozyme J-25 enzyme preparations.

The water-soluble nitrogen was determined in four samples at zero fermentation time. This experiment was used as the control. After 340 minutes of fermentation time, the water-soluble nitrogen was determined again. The difference in these values represented the increase in soluble nitrogen.

Extraction of water-soluble nitrogen was as follows: 50 grams of dough was mixed in the Omni mixer with 100 ml of water for 5 minutes at 6000 r.p.m. The volume was adjusted to 250 ml and an extracted aliquot was centrifuged at 5000 r.p.m. for 15 minutes. The water-soluble nitrogen was determined on the clear portion, using the micro-Kjeldahl method (1).

Evaluating of Bread Quality. The loaf volume was measured immediately as the loaf came from the oven by seed displacement in a loaf volume meter. After cooling for one hour, the bread quality was evaluated, using 100 as a base. Crust color, crumb color, texture, grain, symmetry and break and shred were evaluated, using 10, 10, 20, 20, 10 and 10 scores, respectively, representing the ideal loaf of bread. Score for loaf volume was determined by using the following equation which provided 20 for the maximum

score for the ideal loaf of bread.

$$\text{Score of loaf volume} = \frac{\text{Loaf volume meter reading} - 1000}{100}$$

Crust Color Measurements. Crust color was measured with a Photovolt reflection meter, using a green filter. The suppressed zero method was applied which required a white standard of 100 value and a dark standard of zero value. The reflectance of the sample from the reading with suppressed zero was obtained by using the following formula:

$$r_x = r_d + \frac{g_x (r_1 - r_d)}{100}$$

r_x = Reflectance of sample.
r_d = Reflectance of dark standard.
r_1 = Reflectance of light standard.
g_x = Photovolt reflection meter reading of sample.

Preparation of Carbonyl Free-chloroform. The chloroform was shaken with a saturated solution of sodium bisulfite in water for at least 5 hours. The chloroform layer was distilled, using a 3-foot distillation column packed with glass beads. The fraction boiling at 60-60.5°C. was collected and stored in brown bottles until used.

Preparation of 2,4-Dinitrophenylhydrazine Reagent (2,4-DNPH). One percent (w/v) 2,4-DPNH reagent in 5 N sulfuric acid was prepared by stirring slowly 55.8 ml of concentrated sulfuric acid and the solid reagent, after which the mixture was slowly stirred into 344 ml of water.

Preparation of 2,4-Dinitrophenylhydrazine Derivatives of Pure Carbonyl Compounds. The 2,4-DNPH derivatives of pure

carbonyl compounds were prepared according to Shriner et al. (66). The derivatives were recrystallized twice from 95 percent ethyl alcohol.

Formation and Extraction of 2,4-Dinitrophenylhydrazine Derivatives of Carbonyl Compounds from Bread Crust. A thin layer of the crust from two one-pound loaves of fresh bread (one hour cooling after baking) was removed carefully and ground for 45 seconds in a Waring Blendor. A 50-gram portion of the ground crust was extracted three times with carbonyl free chloroform. The material was blended for 5 minutes with 120 ml of chloroform and allowed to stand for 10 minutes with occasional shaking, after which time the chloroform was removed by suction filtration. The dry residue was extracted with two more 60-ml portions of chloroform. The filtrate, which was collected in a receiver immersed in an ice bath, was added to 400 ml of 1 percent 2,4-DNPH reagent and reacted for 12 hours at 35°C. The chloroform extract of the hydrozone was concentrated under vacuum and adjusted to 50-ml volume.

Determination of Carbonyl Compounds as 2,4-Dinitrophenylhydrazine Derivatives. Standard curves for seven carbonyl compounds were established by extracting known quantities of purified 2,4-DNPH derivatives with 5 ml of 95 percent ethyl alcohol for 20 minutes from Whatman No. 4 filter paper. The wavelengths of maximum absorption for the 2,4-DNPH derivatives were used to measure the absorbance of unknown samples as follows (25):

2,4-Dinitrophenylhydrazones	mu
Formaldehyde	350
Acetaldehyde	356
n-Propionaldehyde	358
n-Butyraldehyde	358
n-Valeraldehyde	358
n-Hexaldehyde	359
n-Heptaldehyde	359

Once the chromatograms were developed, the total amount of 2,4-DNPH in each spot or zone was determined by cutting the spots from the paper chromatograms and extracting for 20 minutes with 5 ml 95 percent ethyl alcohol. The absorbance was determined, using a Beckman Spectrophotometer (Model DU).

Quantity of the free carbonyl compound in question was obtained by using the values in Table 4 which have been calculated from the standard curves. These values were applied to 50 grams of bread which was extracted by chloroform and reacted overnight with 2,4-DNPH reagent. The chloroform layer was adjusted to exactly 50 ml, and 100 ul of the solution was applied to the paper chromatogram. The optical density was determined by extracting each 2,4-DNPH derivative from the paper with 5 ml of 95 percent ethanol.

Paper Chromatography of 2,4-DNPH Derivatives of Carbonyl Compounds. The paper chromatograph method of Piha et al. (50) for separation of the carbonyl compounds was used. Suitable amounts of the chloroform extract were streaked (one inch) on Whatman No. 4 paper. For better separation, it is necessary to keep the streak narrow. This was accomplished by alternate spotting and drying. The paper was immersed in a 1:1 (v/v) mixture of N,N dimethylformamide and absolute ethyl alcohol up to 1 cm

Table 4. Calculation factors for estimation of carbonyl compounds.

Carbonyl compound	:mg 2,4-DNPH derivatives/ : 100 grams bread crust	:mg carbonyl compound/ :100 grams bread crust
Formaldehyde	O.D reading x 48.750	O.D reading x 6.966
Acetaldehyde	O.D reading x 52.222	O.D reading x 10.262
n-Propionaldehyde	O.D reading x 54.285	O.D reading x 13.235
n-Butyraldehyde	O.D reading x 53.333	O.D reading x 15.243
n-Valeraldehyde	O.D reading x 50.000	O.D reading x 16.175
n-Hexaldehyde	O.D reading x 59.375	O.D reading x 21.221
n-Heptaldehyde	O.D reading x 54.500	O.D reading x 21.146

O.D = optical density.

of the streaked edges. The chromatogram was dried for 20 minutes at room temperature (25-36 C.). The paper was equilibrated in the chromatography cabinet for 5 hours and subsequently developed for 4½ hours, using cyclohexane saturated with N,N dimethylformamide as a solvent. For best separation, the room temperature was decreased to 20°C. during equilibrium and maintained at 19 ± 1°C. during development to avoid the evaporation of cyclohexane from the paper. Care had to be taken to keep the chromatography cabinet absolutely air-tight and at nearly constant temperature during the development since slight variation in temperature caused changes in vapor pressures and therefore, in the rate of evaporation of cyclohexane, causing a very diffuse, poorly resolved spot. Under optimum conditions, sharp, distinct zones were obtained.

Determination of 2-Furaldehyde (furfural) and 5-Hydroxymethyl-2-furaldehyde (HMF).

1. Preparation of p-aminodimethylaniline stannous chloride

double salt (P-ADA). P-ADA was prepared by dissolving 5 g (0.029 M) of p-aminodimethylaniline monohydrochloride (Eastman) in 30 ml of hot 95 percent ethyl alcohol and adding 0.53 g (1.029 M) of stannous chloride dehydrate (Fisher reagent). The mixture was cooled to room temperature after which 50 ml of concentrated hydrochloric acid was added. The colorless crystals which formed were filtered, washed rapidly with a small quantity of 95 percent ethyl alcohol and dried for 2 days or more in a vacuum desiccator. The yield was about 7.0 g of p-aminodimethyl-aniline stannous chloride double salt.

2. Preparation of standard curve. Furfural or HMF (0.05 g) was dissolved in 1000 ml benzene (0.05 mg/ml). Different solutions were prepared, ranging from 0.005 mg/ml - 0.05 mg/ml. Five ml of furfural in benzene of each dilution were added to 5 ml of a 0.025 M solution of the P-ADA agent in absolute methanol. The solution was mixed well and allowed to stand at room temperature for 20 minutes for color development. The absorbance was measured at 495 mu in a Beckman Spectrophotometer (Model D U), using a mixture of 5 ml of P-ADA reagent and 5 ml of benzene as a blank.

3. Determination of furfural and HMF. a 10-g sample of the ground crust was extracted five times in an Omni mixer (2 minutes each) at 0°C. with a volume of 25 ml of benzene. The combined benzene extracts were centrifuged for 15 minutes or until the supernatant was clear. The total volume was adjusted to 25 ml and in some cases, anhydrous sodium sulfate was added to the extract to insure a dry benzene extract. Otherwise, a turbid solution with high absorbance was observed. The combined

concentration of HMF and furfural was determined from a 5-ml benzene extract aliquot, according to Linko (35). Five ml of 0.025 M P-ADA reagent in absolute methanol were added to 5 ml of benzene extract aliquot. The solution was mixed well and allowed to stand at room temperature for 20 minutes for color development. The absorbance was compared with the standard curve.

The quantity of furfural and HMF in question was obtained by using the following values which were calculated from the standard curves: Mg furfural and HMF per 100-gram crust = O.D reading x 7.77. These values applied only in case of extracting 10 grams of crust with benzene and adjusting the volume to 25 ml.

Organoleptic and Consumer Preference Test. Two types of investigations were used to determine bread flavor differences by using a combination of consumer preference tests and flavor profile techniques. One-pound loaves of bread were baked on a laboratory scale, employing the same procedure and formula which were employed for the other experiments. The three different enzyme concentrations, zero H.U as control, 150 H.U, and 500 H.U per 700 grams of flour, were used for both papain and Rhozyme J-25 enzyme preparations.

Preference Tests. The loaves were allowed to cool for about one hour after baking. The bread was sliced one-half inch by a commercial slicing machine and samples of two adjacent slices were placed immediately in polyethylene bags and the top of each bag folded down and fastened with a clip. In order to randomize the tests, the samples were staggered so that every possible arrangement was possible. The number of judges was 15 semi-trained

people. The three different samples of bread representing enzyme concentrations were given to the judges one and a half hours before dinner. The judges were told to determine odor first, smelling the lowest-numbered sample first, followed by the highest-numbered sample. The same procedure was used for taste. Water was taken between samples. The judges were told to arrange odor and taste for the three samples as best, intermediate and poorest. The same procedure was used for desirability which is the combination of odor and taste, and it indicated the degree of consumer preference of the sample. The test was repeated five times.

Flavor Profile. This type of organoleptic test was the same as the first type except it was applied for the Rhozyme J-25 enzyme preparation only. The whole loaf of bread was used instead of slices to determine the odor and consumer preference. The loaves were scored on a basis of the strong odor. The number of judges was 10 persons and the test was repeated four times.

Analysis of variance was performed for all data. In case of significant results, a multiple range test was performed to determine the difference between sample flavors and treatments.

RESULTS AND DISCUSSION

Water-soluble Nitrogen in Fermented Dough. The Maillard reaction, producing many intermediates, has been recognized as a means of producing bread flavor. Since the rate of reaction involving condensations of free reducing sugars with the free amino group of amino acids or proteins is sensitive to

concentration of the reactants, the reaction may be controlled by regulating the amount of one or both of the reactants. In the present investigation, the amount of free amino groups in dough was modified by means of proteolytic enzymes. Table 5 and Fig. 1 show the effect of papain and Rhozyme J-25 on water-soluble nitrogen in doughs after fermentation. It is clear that papain was more active than Rhozyme J-25 in the production of soluble nitrogen in a fermenting dough.

Table 5. Effect of papain and Rhozyme J-25 on water-soluble nitrogen in fermented dough.

Enzyme preparation	H.U./700 grams flour			
	0	300	500	1000
	mg N/100 grams fermented dough			
Papain	245	263	271	336
Rhozyme J-25	245	260	264	270

Both enzymes increased the soluble nitrogen up to 500 H.U. Above 500 H.U., papain caused liquefaction of the dough and a great increase in the soluble nitrogen. This indicated a difference in mode of attack on the gluten protein. It would appear that papain contains greater endopeptidase activity than Rhozyme J-25. Major peptide bonds were broken by both enzymes but the shorter peptide chains were hydrolyzed to smaller peptides and peptones by the endopeptidase present in papain but lacking in Rhozyme J-25.

It might be expected that greater amounts of soluble nitrogen would increase the free amino groups capable of reacting with free reducing sugars in the Maillard reaction. The effect of free

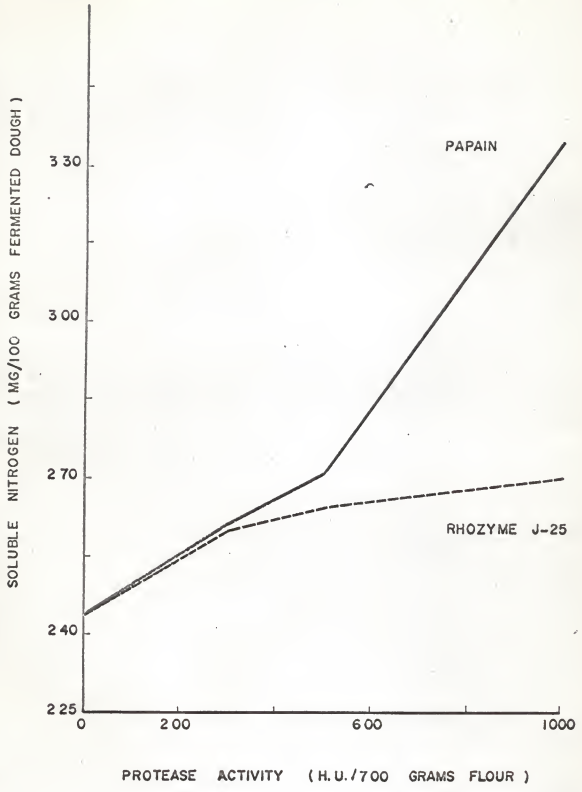

Fig. 1. Effect of papain and Rhozyme J-25 on water-soluble nitrogen in fermented dough.

amino groups, amino acids, peptides and proteins on the Maillard reaction and production of carbonyl compounds in baked bread has been demonstrated by many investigators. Linko and Johnson (35) observed the marked decreases in free amino acids of crust, together with the formation of several aldehydes. They suggested the importance of the Maillard-type browning in flavor production. Similar observations regarding the participation of amino acids in non-enzymatic crust browning and in the subsequent formation of carbonyl compounds in bread have been reported by Kretovich and Tokareva (32). The dipeptides also significantly increased the carbonyl compounds in baked bread (57). Amino acids act as precursors for several aldehydes during baking (28, 36, 60). It has also been reported that the addition of amino acids or non-fat dry milk solids increases crust browning (6, 79).

Evaluation of Bread Quality. There are various observations which suggest that proteinase plays an important role in bread production (21, 23, 27, 46). Miller and Johnson (46) concluded that enzymes can be used to modify dough properties as well as to improve the flavor-imparting characteristics of the ferment. Johnson and Miller (22) reported that proteinases break the protein chains at the ptptide linkage, resulting in a more extensible dough which expands with greater ease and exhibits greater pan flow. In addition to the desirable effect of amylase and protease on dough properties, these enzymes, in proper quantities, improve the properties of bread. Flour supplemented with both alpha-amylase and proteases improve the grain, texture and loaf volume. Excessive proteolysis in dough, however, can be

very serious and may result in coarse grain and texture and impaired loaf volume. Also, if the proteolysis is excessive, by virtue of a large concentration of proteolytic enzymes, a liquefaction of the dough may result. Therefore, determining the optimum level of papain and Rhozyme J-25, protease activity is necessary in order to control bread quality.

The data in Tables 6 and 7 show the effect of various levels of papain and Rhozyme J-25 on the specific volume of bread. Papain, up to 500 H.U., increased the specific volume of bread. Higher enzyme concentration impaired the volume. This was due apparently to lack of gas retention resulting from extensive hydrolysis of the gluten protein. Rhozyme J-25 contained, in addition to proteinases, some alpha-amylase and this may have contributed to the increase in loaf volume. It was observed that loaf volume increases were generally accompanied by greater browning of the crust.

The effects of papain and Rhozyme J-25 on other bread characteristics are summarized in Tables 8 and 9. Both enzyme preparations up to 500 H.U. per 700 grams of flour improved the other bread characteristics. Papain caused maximum improvement of bread quality at the 300 H.U. level. At higher enzyme levels, the quality of bread decreased and at concentrations of 700 and 1000 H.U., bread quality characteristics were impaired extensively. Rhozyme J-25 was not injurious to the quality of the bread even up to 1000 H.U. Excess protease activity caused undesirable bread, and proper supplementation with Rhozyme J-25

Table 6. Effect of papain on specific volume of bread.

	H.U./700 grams of flour						
	0	75	150	300	500	700	1000
Wt. of loaf (gm)	517	412	408	404	410	495	499
Volume (cc)	2900	2925	3000	3000	3000	2150	1650
Specific volume (cc/gm)	6.95	7.09	7.35	7.42	7.32	4.24	3.30

Table 7. Effect of Rhozyme J-25 on specific volume of bread.

	H.U./700 grams of flour						
	0	75	150	300	500	700	1000
Wt. of loaf (gm)	407	416	407	405	407	405	405
Volume (cc)	2800	2900	3000	2975	3000	3000	3000
Specific volume (cc/gm)	6.88	6.97	7.37	7.37	7.37	7.40	7.40

Table 8. Effect of papain on bread quality.

	H.U./700 grams of flour						
Max.	0	75	150	300	500	600	1000
Crust color	10	8	8	7	8	9	9	9
Crumb color	10	7	7	7	7	8	3	3
Symmetry	10	8	9	8	8	8	3	1
Break & shred	10	6	7	8	8	6	0	0
Grain	20	14	14	15	16	16	7	5
Texture	20	15	16	17	17	16	8	5
Volume	20	19	19	20	20	20	11	6
Total score	100	77	81	84	85	83	38	25

Table 9. Effect of Rhozyme J-25 on bread quality.

		H.U./700 grams of flour						
	Max.	0	75	150	300	500	600	1000
Crust color	10	8	8	7	8	9	9	9
Crumb color	10	8	8	8	8	8	8	8
Symmetry	10	8	8	8	9	9	8	9
Break & shred	10	7	7	6	9	8	8	8
Grain	20	15	15	16	15	15	15	15
Texture	20	15	16	16	17	17	17	17
Volume	20	18	19	20	20	20	20	20
Total score	100	79	81	81	86	86	85	86

and papain may produce loaves that are symmetrical, soft, with grain and texture characteristics that are superior.

Since uniform levels of papain and Rhozyme J-25 protease activity show different effects on bread characteristics, as indicated in Figs. 2 and 3, it was clear that the Ayre-Anderson method for evaluating the two enzyme preparations did not reflect, necessarily, the effects of the enzymes on bread quality. Therefore, the optimum level for protease supplementation may vary, depending upon the type of protease system. Research on the use of fungal preparations in baking (46) has revealed that optimum levels for protease supplementation are within somewhat a narrower range than that for amylase. The requirements of different flours for enzyme supplementation are not related to the flour source, protein content or the amylograph viscosity of the unsupplemented flour. Therefore, the requirement for protease supplementation, in particular, must be determined by the baking test. In this study, papain at 300 H.U. per 700 grams flour and up to 1000 H.U. of Rhozyme J-25 produced the best quality of bread.

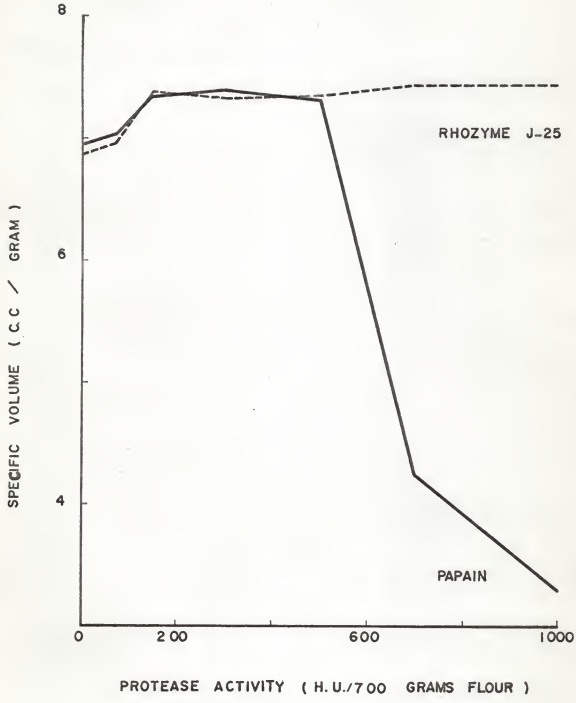

Fig. 2. Effect of papain and Rhozyme J-25 on specific volume of bread.

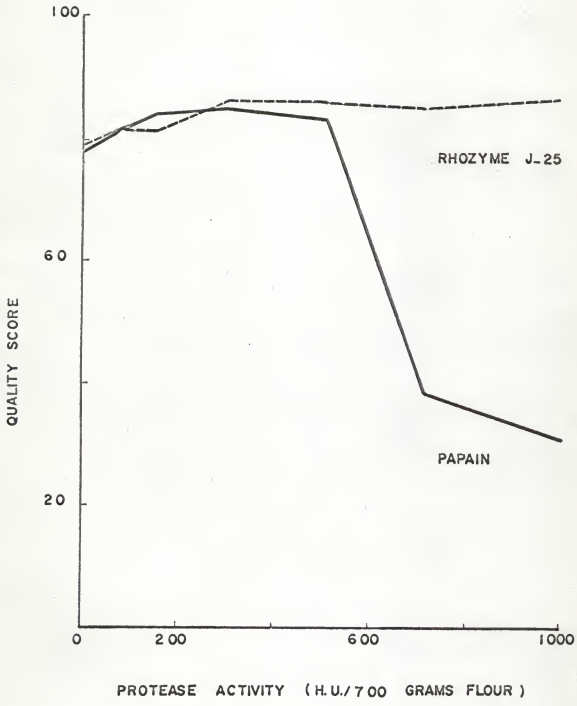

Fig. 3. Effect of papain and Rhozyme J-25 on bread quality.

Crust Color Intensity. The relationship between crust color and bread flavor has been reported by many investigators. Baker (4) found that neither normally fermented bread baked without a brown crust, nor improperly fermented bread baked with a crust had good flavor. Their work suggested that such flavor arises during crust formation. Similar conclusions were reached by Thomas and Rothe (71) who found crust-forming conditions essential for full flavor production. Measurement of the intensity of crust color would, therefore, be important.

Table 10 and Fig. 4 show the effect of papain and Rhozyme J-25 on the photovolt reflectance reading of bread crust.

Table 10. Effect of papain and Rhozyme J-25 on bread crust color (% reflectance).

Enzyme preparation	H.U./700 grams flour						
	0	75	150	300	500	700	1000
Papain	29.0	25.3	21.8	19.7	16.0	30.0	33.5
Rhozyme J-25	28.0	27.0	25.7	25.0	25.0	23.7	25.0

The reflectance values, the average of six readings, are inversely related to color intensity. Slight but steady increase in crust color was observed as the Rhozyme J-25 concentration was increased. Papain intensified crust color up to 500 H.U. per 700 grams flour. Since the rate of reaction of the condensation of sugar with the amino group, i.e. Maillard reaction, is affected by concentration of the reactants, it might be expected that the increase in free amino groups, as a result of proteolysis in the dough, would increase the crust color. Similar results have been

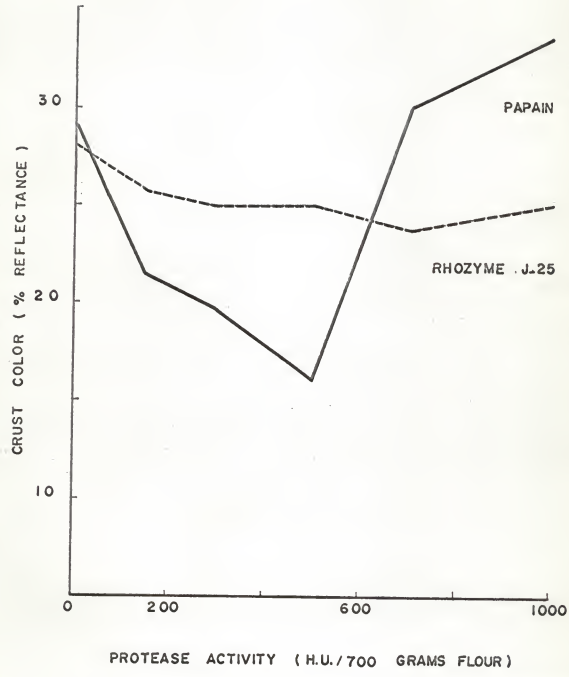

Fig. 4. Effect of papain and Rhozyme J-25 on bread crust color.

reported by Haney (13) who indicated that supplementing with 5.0 percent dextrose and increasing amounts of tryptophan increased the darkness of the bread crust. The lack of amino acids in model systems prevented formation of brown pigments and the creation of intermediate compounds of the Maillard reaction. Bertram (6) concluded that normal crust color is a result of the reaction between free amino groups and reducing sugars. The addition of amino acids markedly enhanced the crust color over that obtained without added amino acids (57, 64). Also, it was observed at 700 H.U. papain activity per 700 grams flour that the crust color decreased markedly and reached a minimum crust color at the 1000 H.U. level. These results can be explained on the basis of Maillard reaction. The Amadori rearrangement involves isomerization of the N-substituted glycosylamine product of condensation of free amino groups with reducing sugars to form 1-amino-1-deoxy-2-ketose, which when heated during baking, undergoes dehydration and forms carbonyl compounds by the Schiff base and Strecker degradation reactions. The carbonyl compounds can further react with more amino acids or free amino groups to form aldimines or undergo an aldol condensation. Both reactions lead to the production of polymerized pigments. Also, the Amadori rearrangement product may break down to form sugar fisson products such as propionaldehyde and diacetyl which can react with amino acids and undergo the Strecker degradation or form aldimines which polymerize to form the melanoidin pigments. Since the production of carbonyl compounds decreased markedly when over 500 H.U. per 700 grams flour of papain was used, the decrease in

brown pigments in the crust at 700 and 1000 H.U. levels of papain was expected.

Production of Carbonyl Compounds. Furfural and HMF were determined quantitatively from benzene extracts of bread crust. The following concentrations of papain and Rhozyme J-25 were used: 0, 75, 150, 300, 500, 700 and 1000 H.U. per 700 grams flour. The effect of papain and Rhozyme J-25 on the production of furfural and HMF in bread crust is summarized in Table 11 and Fig. 5. It is apparent that the increase of furfural and HMF in the bread crust was proportional to the papain enzyme concentration up to 500 H.U. per 700 grams flour. At 700 and 1000 H.U. levels, the concentration of these compounds decreases rapidly. These data indicate that Rhozyme J-25 had relatively little effect on the production of furfural and HMF in the bread crust. Furfural content proportional to sugar and protein content of various baked products have been reported by Rotsch (58, 59). Furfural was higher in well-baked, dark samples (59). Furfural is believed to be one of the most stable aromatic compounds (58). Rothe and Thomas (61) concluded that only iso-butanal, iso-valeraldehyde and furfural are important in bread flavor.

The data in Table 12 and Fig. 6 illustrate the effect of papain and Rhozyme J-25 on the production of total carbonyl compounds. The total carbonyl compounds in bread crust increased with the papain enzyme concentration up to 500 H.U./700 grams flour. At higher enzyme levels, the production of carbonyl compounds decreased, reaching a minimum at the 1000 H.U. level. On the other hand, Rhozyme J-25 caused a slight increase in the

Table 11. Effect of papain and Rhozyme J-25 on production of furfural and HMF in bread crust.

H.U./700 grams flour	Papain	Rhozyme J-25
	mg/100 grams bread crust	
0	8.20	8.22
75	10.92	7.91
150	13.01	8.23
300	15.21	9.11
500	17.90	9.51
700	8.02	8.80
1000	6.81	8.21

Table 12. Effect of papain and Rhozyme J-25 on production of total carbonyl compounds in bread crust.

H.U./700 grams flour	Papain	Rhozyme J-25
	mg/100 grams bread crust	
0	17.92	17.99
75	22.25	19.81
150	35.10	20.52
300	32.37	23.96
500	34.15	23.94
700	18.49	23.38
1000	14.35	23.49

carbonyl compound development as the enzyme activity increased up to 1000 H.U. per 700 grams flour. These data correlated well with the effect of the enzymes on crust color and specific volume of the bread. Therefore, a relationship between crust color formation and total carbonyl compounds appears to exist. This was most obvious in case of furfural and HMF. Linko et al. (36) reported that a relationship existed between crust color and concentration of carbonyl compounds. The crust browning of bread has been recognized as a source of bread flavor (Haney, 13).

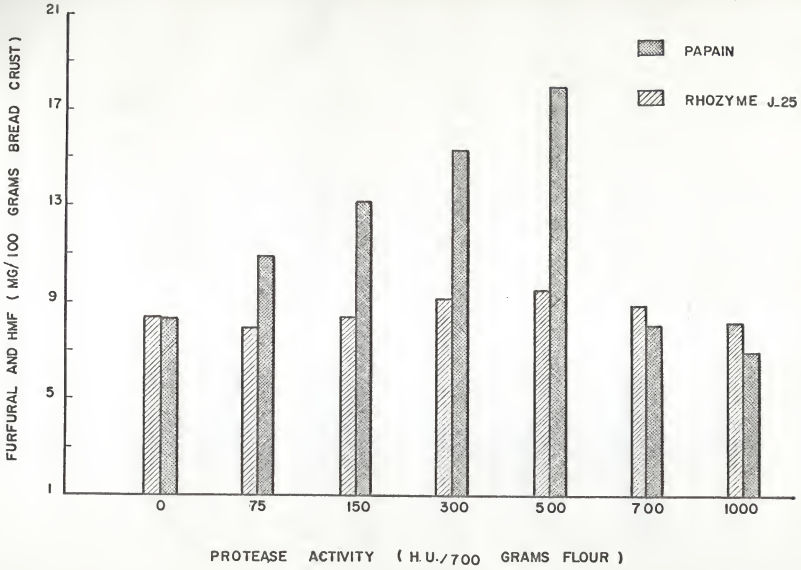

Fig. 5. Effect of-pepain and Rhozyme J-25 on production of furfural and HMF in bread crust.

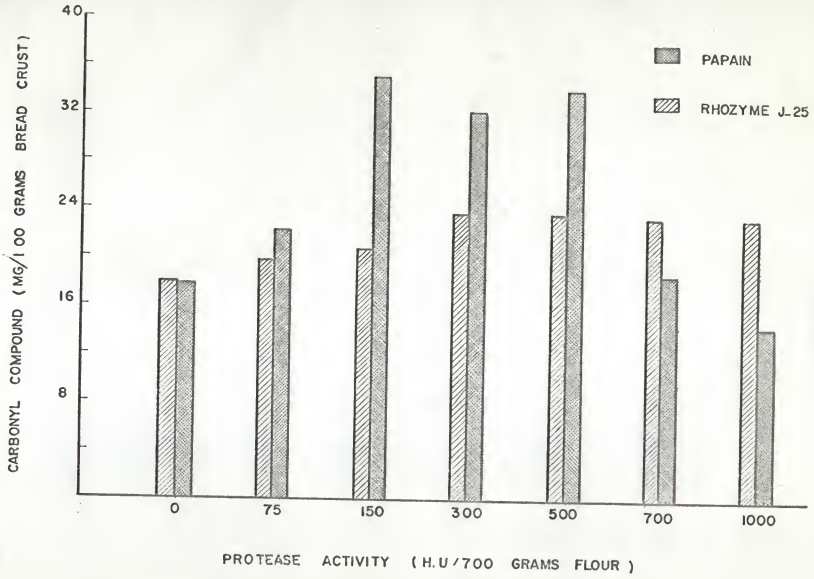

Fig. 6. Effect of papain and Rhozyme J-25 on production of total carbonyl compounds in bread crust.

Haney also indicated that the flavor of cookies was markedly improved as the extent of browning increased. Similar conclusions were reached by Thomas and Rothe (71) who found crust-forming conditions essential for full bread flavor production.

Increase in total carbonyl compounds with increments of enzymatic activity in bread dough can be explained on the basis of increasing the water-soluble nitrogen as a result of proteolysis in fermented doughs (Table 5 and Fig. 1) and subsequent increase of the free amino groups. The rate of reaction of the condensation of sugar with free amino groups, i.e. Maillard type reaction, is related to the concentration of the reactants. Browning and production of carbonyl compounds may be increased during baking as a result of increased amounts of free amino groups in a fermented dough.

Kiely (28) found that the kind of sugar has little effect on aroma but did have a considerable effect on the rate of reaction. The aroma was controlled by amino acids. Rothe (60) reacted 14 individual amino acids with xylose and in each case an aliphatic aldehyde as well as furfural was formed and he demonstrated further that these reactions take place in the crust during bread baking. It has been reported that the addition of amino acids or non-fat dry milk solids in dough increases crust browning (6, 79). Salem (64) reported reaction of water-soluble proteins produced carbonyl compounds which could be separated into seven distinct bands by paper chromatography. Salem (64) and Rooney (57) demonstrated that addition of amino acids and peptides to bread increased the total carbonyl compounds. They illustrated

the importance of the Maillard reaction in crust browning production and formation of carbonyl compounds.

The decrease in total carbonyl compounds at high levels of papain activity may be due to a combination of several factors. Since the bread volume was impaired at 700 and 1000 H.U. per 700 grams flour, less surface was subjected to heat during baking, causing less browning and less production of carbonyl compounds. Johnson et al. (26) have reported that excessive proteolytic activity in dough impaired loaf volume due primarily to lack of gas retention. As was indicated by the data in Table 6 and Fig. 2, at higher papain levels, the specific volume was impaired as a result of extensive hydrolysis of gluten proteins. Also, the free amino groups may react further with the carbonyl compounds which are produced from the Maillard reaction during baking to form products containing a carbon-nitrogen bond. Moreover, the slight acidity of a fermented dough favors this type of reaction. These products do not react with the 2,4-DNPH reagent, resulting in less recovery of carbonyl compounds of bread at this high level of enzyme activity.

The quantitative determination of individual carbonyl compounds is important since each aldehyde has a distinct effect on bread flavor. Baker (4) attached special importance to pyruvaldehyde, iso-aldehydes, furfural and diacetyl on bread flavor. Rothe and Thomas (61) concluded that only iso-butanan, isovaleraldehyde and furfural were important in bread flavor.

Paper chromatography, using the cyclohexane-N,N-dimethylformamide solvent, was used for quantitative estimation of the

2,4-DNPH derivatives of carbonyl compounds which were isolated in the chloroform extract from crust of bread treated with 0, 75, 150, 300, 500, 700 and 1000 H.U. of papain and Rhozyme J-25 per 700 grams flour. The carbonyl compounds were divided into seven groups on the basis of their R_{val} (57). R_{val} is the ratio of distances moved by the unknown 2,4-DNPH and by the 2,4-DNPH of n-valeraldehyde, using N,N-dimethylformamide-cyclohexane paper chromatography systems as indicated in Table 13. These bands contained the following 2,4-DNPH derivatives:

Group 1: Formaldehyde only.

Group 2: Acetaldehyde and benzaldehyde but expressed as acetaldehyde.

Group 3: Propionaldehyde, acetone and phenylacetaldehyde but expressed as propionaldehyde.

Group 4: n-Butyraldehyde, iso-butyraldehyde and methyl-ethylketone but expressed as n-butyraldehyde.

Group 5: n-Valeraldehyde, iso-valeraldehyde and 2-methyl-butanal but expressed as n-valeraldehyde.

Group 6: n-Hexaldehyde, 2-hexanone and 2-methylpentanal but expressed as n-hexaldehyde.

Group 7: n-Heptaldehyde, 3-heptanone, 2-ethylhexanal and 4-heptanone but expressed as n-heptaldehyde.

R_{val} values varied slightly with each determination. Thus, it was necessary to have knowns of 2,4-DNPH derivatives of pure formaldehyde, acetaldehyde, propionaldehyde, n-butyraldehyde, n-valeraldehyde, n-hexaldehyde and n-heptaldehyde mixture and unknowns side by side on the same chromatogram. However, the

Table 13. R_{val} values of 2,4-DNPH derivatives of pure carbonyl compounds (N,N-dimethylformamide cyclohexane chromatogram systems).

Compound	:	R_{val} values
Formaldehyde		0.31
Acetaldehyde		0.45
Benzaldehyde		0.42
Propionaldehyde		0.67
Phenylacetaldehyde		0.63
Acetone		0.63
n-Butyraldehyde		0.86
Methylethylketone		0.86
iso-Butyraldehyde		0.40
n-Valeraldehyde		1.00
iso-Valeraldehyde		1.03
2-Methylbutanal		1.00
n-Hexaldehyde		1.13
2-Hexanone		1.15
2-Methylpentanal		1.15
n-Heptaldehyde		1.23
3-Heptanone		1.32
2-Ethylhexanal		1.30
4-Heptanone		1.29

R_{val} value of one 2,4-DNPH derivative relative to each other remained fairly constant for all determinations.

The effect of papain on production of carbonyl compounds is summarized in Table 14 and Fig. 7. Propionaldehyde was produced in highest concentration, reaching maximum with 150 H.U. per 700 grams flour. This was followed by acetaldehyde and butyraldehyde. Linko (34) reported that acetone and HMF were the major components, with others present in minor quantities in bread. Formaldehyde production was not influenced by proteolysis. Figure 8 shows that n-valeraldehyde increased slightly. n-Hexaldehyde reached its highest production with 500 H.U. per 700 grams flour but decreased rapidly with 700 and 1000 H.U. n-Heptaldehyde

Table 14. Effect of papain on production of some carbonyl compounds in bread crust.

H.U./700 g flour	Form-aldehyde	Acet-aldehyde	n-Propion-aldehyde acetone	n-Butyr-aldehyde iso-Butyr-aldehyde Methyleth-yl ketone	n-Valer-aldehyde iso-Valer-aldehyde 2-Methyl-butanal	n-Hex-aldehyde 2-Hexanone 2-Methyl-pentanal	n-Hept-aldehyde 3-4-Hep-tanone 2-Ethyl-hexanal	Total
				mg/100 grams of fresh weight				
0	0.63	1.45	1.95	1.45	0.25	1.13	1.86	9.72
75	0.69	1.66	2.55	1.99	1.17	1.19	2.12	11.33
150	0.96	4.82	7.84	3.84	1.05	0.59	2.29	22.09
300	0.77	2.21	4.96	2.84	1.33	1.83	3.22	17.16
500	0.75	1.79	3.89	2.21	1.24	2.95	3.42	16.25
700	0.56	1.41	1.38	1.13	0.78	2.25	2.96	10.47
1000	0.50	0.92	1.22	0.92	0.53	0.70	2.75	7.54

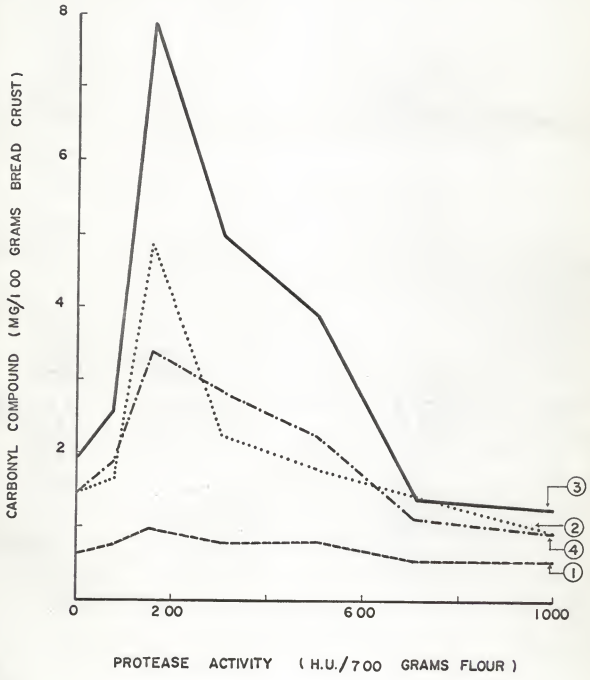

Fig. 7. Effect of papain on production of (1) formaldehyde, (2) acetaldehyde, (3) propionaldehyde and (4) n-butyraldehyde in bread crust.

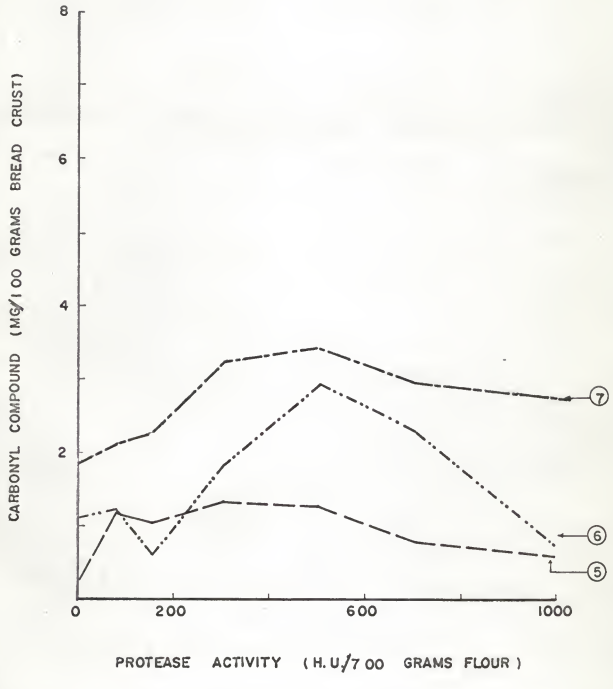

Fig. 8. Effect of papain on production of (5) n-valeraldehyde, (6) n-hexaldehyde and (7) n-heptaldehyde in bread crust.

increased up to 500 H.U. per 700 grams flour, followed by a slight decrease at higher levels of enzyme activity.

Thozyme J-25 caused a slight increase in the carbonyl compound development as the enzyme activity increased (Table 15 and Figs. 9 and 10). n-Heptaldehyde was the predominant carbonyl compound produced in crust of bread made with Rhozyme J-25. Formaldehyde was not influenced by increasing the Rhozyme-25 concentration. Production of carbonyl compounds in baked bread has been reported by many investigators. Thomas and Rothe (70, 71) listed ten aldehydes which they found as by-products of the Maillard reaction. Each was specifically associated with a starting amino acid. The end-products were furfural, HMF, acetaldehyde, iso-butanal, iso-valeraldehyde, 2-methylbutanal, methanal, phenylacetaldehyde, 2-hydroxypropional and pyruvaldehyde. These compounds arise as by-products of the Maillard reaction during baking as a result of the Strecker degradation of specific amino acids.

Evaluating of Bread Flavor. The primary objective of bread flavor evaluation was to determine whether papain and Rhozyme J-25 enzyme action in fermenting dough influenced the production of carbonyl compounds in bread. Quantitative analysis of carbonyl compounds in bread crust showed that the total carbonyl compounds were increased as the papain and Rhozyme J-25 concentration increased up to 500 H.U. per 700 grams flour. Increase in the total carbonyl compounds was slight in case of Rhozyme J-25 and at 150 H.U. of papain per 700 grams flour. The bread crust contained less furfural and HMF but more of the other carbonyl

Table 15. Effect of Rhozyme J-25 on production of some carbonyl compounds in bread crust.

H.U./700 g flour	Form-aldehyde	Acet-aldehyde	n-Propion-aldehyde acetone	n-Butyr-aldehyde iso-Butyr-aldehyde Methyleth-yl ketone	n-Valer-aldehyde iso-Valer-aldehyde 2-Methyl-butanal	n-Hex-aldehyde 2-Hexanone 2-Methyl-pentanal	n-Hept-aldehyde 3-4-Hep-tanone 2-Ethyl-hexanal	Total
				mg/100 grams of fresh weight				
0	0.63	1.45	1.95	1.47	0.27	1.13	1.86	9.77
75	0.80	1.54	1.92	1.60	1.21	1.34	3.49	11.90
150	0.86	1.75	1.88	1.63	1.00	1.15	4.02	12.99
300	1.02	2.17	2.44	1.81	1.49	1.23	4.69	14.85
500	0.98	2.04	2.26	1.92	1.35	1.42	4.46	14.43
700	0.90	1.99	2.03	1.83	1.52	1.55	4.76	14.58
1000	1.95	1.90	2.12	1.93	1.46	1.87	5.05	15.28

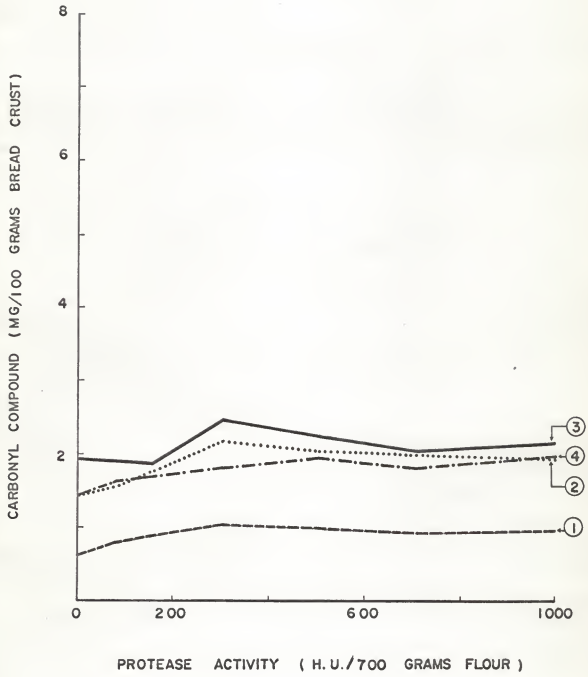

Fig. 9. Effect of Rhozyme J-25 on production of (1) formaldehyde, (2) acetaldehyde, (3) propionaldehyde and (4) n-butyraldehyde in bread crust.

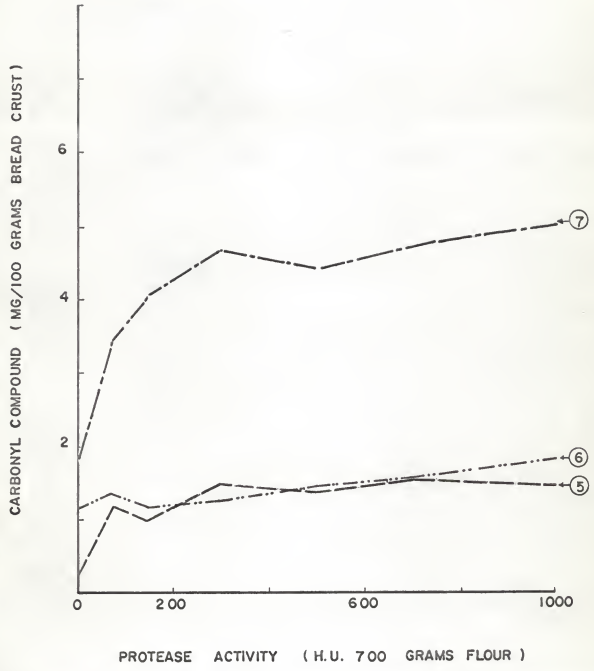

Fig. 10._ Effect of Rhozyme J-25 on production of (5) n-valeraldehyde, (6) n-hexaldehyde and (7) n-heptaldehyde in bread crust.

compounds. At 500 H.U. of protease activity, the bread crust contained more furfural and HMF and less of the other carbonyl compounds. At both 500 and 700 H.U. levels, the amount of total carbonyl compounds in the crust was about equal but about twice as great as in bread without enzyme supplement. On the basis of these differences in carbonyl compounds as a result of enzyme activity, the concentrations of 150 and 500 H.U. per 700 grams flour of papain and Rhozyme J-25 were used for organoleptic tests. A combination of consumer preference tests and flavor profile techniques were used for evaluating bread flavor. For statistical analysis, the data were grouped for each test separately. In order to do the analysis of variance, which has been recommended for this type of organoleptic test (11), normal score transformation for the grouped data was accomplished by assigning the following values: +0.85, 0 and -0.85 for respective ranks. Summation of values for every test was indicated as total calculated score. "F" value was calculated (19) to test whether or not the decision of the judges was significant. Data of bread taste, and aroma organoleptic tests of papain and Rhozyme J-25 treatments, including total calculated scores and calculating "F" values, are summarized in Tables 16, 17 and 18.

The "F" values were used to test significance of the calculated "F" values at the 5 percent level. All the results by analysis of variance were insignificant except for papain bread aroma. These results indicate that neither the effect of the three treatments with Rhozyme J-25 on bread aroma and taste nor the effect of treatment of papain on bread taste were significant.

Table 16. Grouped data of bread aroma, taste and desirability organoleptic tests of papain enzyme preparations.

	Odor test			Taste test			Desirability test		
	Enzyme preparation concentration H.U./700 grams flour								
Rating order	0	150	500	0	150	500	0	150	500
	Score numbers for tested bread slices								
Best	19	24	34	29	17	29	30	17	28
Intermediate	21	26	24	23	30	22	22	32	21
Poorest	35	25	17	23	28	24	23	26	26
Total calculated scores	-13.5	-0.85	+14.45	+5.10	-9.35	+4.25	+5.95	-7.65	+1.7
Calculated F value		3.76			1.31			0.89	

Table 17. Grouped data of bread aroma, taste and desirability organoleptic tests for three concentrations of Rhozyme J-25.

Rating order	Odor test			Taste test			Desirability test		
	Enzyme preparation concentration H.U./700 grams flour								
	0	150	500	0	150	500	0	150	500
	Score numbers for tested bread slices								
Best	32	20	25	26	23	28	23	27	23
Intermediate	21	25	25	28	30	23	32	15	31
Poorest	22	30	25	21	32	24	20	33	21
Total calculated scores	+8.5	-8.5	0	+4.25	-7.65	+3.40	+2.55	-4.25	+1.7
Calculated F value		0.131			0.81			0.25	

Table 18. Grouped data of bread aroma and desirability organoleptic tests for three concentrations of Rhozyme J-25.

Rating order	Odor test: Enzyme preparation concentrations H.U./700 grams flour: 0	150	500	Taste test: Enzyme preparation concentrations H.U./700 grams flour: 0	150	500
	Score numbers for tested whole loaf of bread					
Best	5	10	15	8	7	15
Intermediate	8	11	11	11	13	8
Poorest	17	9	4	11	12	7
Total calculated scores	-10.20	+0.85	+9.35	-2.55	-4.25	+6.8
Calculated F value		2.37			0.74	

In the case of papain, the bread aroma was significantly different at the 1 percent level. Therefore, Rhozyme J-25 in concentrations up to 500 H.U. level per 700 grams flour had an insignificant effect on bread flavor, but papain influenced the bread aroma up to 500 H.U. per 700 grams flour. Papain exhibited a greater effect on bread aroma at 500 H.U. than at the 150 H.U. level. Miller and Johnson (46) reported that enzymes can be used to improve the flavor-imparting quality. Also, it was clear that carbonyl compounds affected bread aroma, but did not affect bread taste which seems to be affected by other factors rather than carbonyl compound production. Furfural and HMF have a significant influence on bread aroma. The effect of carbonyl compounds on bread flavor has been demonstrated by many investigators. Thomas and Rothe (69, 71) related the flavor of different types of bread to the amount of carbonyl compounds. The effect of furfural and HMF on bread flavor has been reported by Komm and Lehman (31).

Kretovich and Tokareva (32) earlier had related bread flavor to the total aldehyde content. Carlin (7) demonstrated the significance of carbonyl compounds in bread flavor by the positive correlation of organoleptic tests and total aldehyde content. Also, association of carbonyl compounds with bread aroma and flavor has been reported by Salem (64) and Rooney (57). It is generally accepted that aldehydes and ketones are very important in the production of bread flavor. In bread made by the sour dough process, the aroma was related to furfural content (58). In breads of different types, a relationship existed between the total aldehyde content and flavor (77).

SUMMARY AND CONCLUSIONS

An investigation of the effect of various concentrations of papain and Rhozyme J-25 enzyme preparations on bread quality, bread crust color, production of carbonyl compounds and bread flavor was conducted. At comparable levels of proteases activity in bread, papain, up to 500 H.U. per 700 grams flour, showed more effect on bread crust color, bread quality, production of carbonyl compounds and bread flavor than Rhozyme J-25. Therefore, the Ayre-Anderson method for evaluating the two enzyme preparations does not necessarily reflect the effects of the enzymes on bread characteristics. Papain, at 300 H.U. per 700 grams flour and up to 1000 H.U. of Rhozyme J-25, produced the best quality of bread.

A relationship between crust color and total carbonyl compound production appears to exist. This was most obvious in the case of furfural and hydroxymethylfurfural (HMF). Also, it was

observed that loaf volume increases were generally accompanied by greater browning of the bread crust. Rhozyme J-25, up to 1000 H.U. per 700 grams flour, showed a slight increase in total carbonyl compound production in bread crust. Furfural, HMF and propionaldehyde were predominant in bread crust treated with papain up to 500 H.U. per 700 grams flour.

Increase in total carbonyl compounds and crust color with increments of proteases in bread is associated with the increase of free amino groups in fermented dough. Increased amounts of available amino groups caused a more predominant Maillard reaction. At higher levels of papain activity, production of carbonyl compounds, crust color and bread quality was decreased markedly. This likely was due to extensive hydrolysis of gluten protein, resulting in less bread volume with less surface area subject to the heat during baking. Decrease of carbonyl compound production decreased the rate of brown melanoidin pigment formation, causing less crust color. Formaldehyde production was not influenced by increasing the papain or Rhozyme J-25 activity in bread.

Rhozyme J-25, in concentrations up to 500 H.U. per 700 grams flour, had an insignificant effect on bread flavor, but papain influenced the bread aroma at 150 and 500 H.U. levels. Also, it was clear that carbonyl compounds affected bread aroma but did not affect bread taste which seems to be affected by other factors rather than carbonyl compound production. Furfural and HMF had a significant influence on bread aroma.

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. John A. Johnson, major professor, for his valuable advice and encouragement throughout the course of this investigation and for the preparation of this manuscript. Gratitude is also extended to the supervisory committee and other members of the faculty and staff of the Flour and Feed Milling Industries Department, who were willing to give of their time for counsel and advice during the course of this work.

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EFFECT OF PROTEASE ENZYMES ON BREAD FLAVOR

by

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B. S., Ain Shams University,
Egypt, U. A. R., 1961

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Flour
and Feed Milling Industries

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1966

An investigation of the effect of papain and Rhozyme J-25 proteases activity on bread quality, bread crust color, production of carbonyl compounds and bread flavor was conducted. Proteases activity was determined by the Ayre-Anderson method. Levels of 0, 75, 150, 300, 500, 700 and 1000 H. U. of both enzymes per 700 grams flour were used in bread. Crust color was measured with a photovolt reflection meter. Organoleptic and consumer preference techniques were used for evaluating bread flavor. Furfural and HMF were determined quantitatively from benzene extracts of bread crust by measuring color developed in reaction with P-aminodimethylaniline stannous chloride double salt (P-ADA). Other carbonyl compounds were isolated as their 2,4-DNPH derivatives and were quantitatively determined by paper chromatography, using the cyclohexane-M,N-dimethylformamide system.

At comparable levels of proteases activity in bread, papain, up to 500 H.U. per 700 grams flour, showed more effect on bread crust color, bread quality, production of carbonyl compounds and bread flavor than Rhozyme J-25. Therefore, the Ayre-Anderson method for evaluating the two enzyme preparations does not reflect, necessarily, the effects of the enzymes on bread characteristics. Papain, at 300 H.U. per 700 grams flour and up to 1000 H.U. of Rhozyme J-25, produced the best quality of bread.

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