AN INVESTIGATION OF COMMERCIAL BACTERIAL AND FUNGAL PREPARATIONS AS ALPHA-AMYLASE SUPPLEMENTS IN BAKING

by

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B. S., Kansas State College
of Agriculture and Applied Science, 1948

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Milling Industry

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1949

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INTRODUCTION

The ability of malted cereals to impart desirable characteristics to bread has been recognized for hundreds of years. In recent decades great advances have been made in understanding the use and action of malt supplements. The desirable characteristics have been shown to occur as the result of the action of certain enzyme systems which are present in the added supplement. Johnson, Dirks and Shellenberger (11) evaluated amylase supplements as follows:

> Present knowledge of the active enzyme systems present in bread dough indicates that three, namely, beta- and alpha-amylase and proteolytic enzyme are concerned with the malt supplementation problem. Beta-amylase is present in excess in normal flour while alpha-amylase is added chiefly through the use of malt supplements. Proteolytic enzyme usually accompanies the amylase enzymes in malt supplements.

Several sources of alpha-amylase and proteolytic enzyme systems have been recognized. Germinated cereals are the most common source of diastatic supplements. Barley and wheat malt, however, are the only flour supplements used extensively. Rye malt has been used to a minor extent, and Knaan and Sandstedt (14) reported there is no obvious reason why the malts of other cereals including oats, sorghum, or maize could not be employed if such supplements would comply with existing food regulations. It was shown by Knaan (13) that all these cereals produce alpha-amylase on germination.

Other possible sources of the enzyme systems present in malt supplements include those produced by bacteria and fungi. Chiefly

through the efforts of Takamine, commercial production of enzyme preparations from microbial sources was developed (27). There has been extensive commercial production of an alpha-amylase by the growth of selected strains of Bacillus subtilis or Bacillus mesentericus. Another commercial amylase preparation has been produced by growing a selected strain of Aspergillus oryzae on suitable media. The amylases generally produced by these organisms have been found to be of the alpha type. Kneen and Sandstedt (14) stated that the use of a purified sterile preparation of mold-amylase concentrate as a flour supplement should give results similar to those obtained by using malt alpha-amylase. Data by Green (6), Read and Haas (21), and Miller and Johnson (18) suggested that mold amylase may be used in baking.

Should alpha-amylase supplements from sources other than cereals prove desirable in the baking industry, the utilization of these preparations would be determined in part by the economics of their production. The approval of the Pure Food and Drug Administration must be secured before bacterial or fungal alpha-amylase preparations may be used for flour supplementation.

A number of problems are involved in the evaluation of new alpha-amylase sources. Kozmin (16) and Proskuryakov, Grinberg and Kozhevnikova (20) have shown that excessive malt alpha-amylase supplementation caused an inelastic and sticky crumb. With increasing alpha-amylase supplementation there was a sharp increase in dextrin formation with a corresponding loss of starch. The use of 0.005 percent potassium bromate gave a slight improvement

and decrease in water extract and dextrins. Lactic acid also had an improving effect when malt extract was used. Miller and Johnson (18) observed no stickiness of the crumb when using an aqueous extract equivalent to 6 percent malted wheat flour.

Kneen and Sandstedt (13) reported that bacterial amylases have a considerably higher degree of thermostability than malt alpha-amylase. These workers (13) postulated that starch breakdown in the oven may be excessive, and some of the alpha-amylase may even remain active throughout the baking period and cause liquefaction of the gelatinized starch after the bread is removed from the oven. This would result in undesirable sticky and gummy bread crumb characteristics similar to those associated with "ropy" bread. Tilden and Hudson (26), however, have reported that alpha-amylases from different bacterial strains may differ in thermostability. These workers (26) found that the alpha-amylase produced by Bacillus macerans showed no marked inactivation when heated at 50°C. for one hour, while the alpha-amylase produced by Bacillus polymyxa was inactivated by the same treatment.

Differences in the behavior of bacterial alpha-amylase as compared with the behavior of malt alpha-amylase were demonstrated by Hopkins and Kulka (7). In the later stages of starch hydrolysis differences were not due to lack of stability of the bacterial alpha-amylase nor to the presence of inhibiting substances. These workers (7) suggested that the difference may be due to less affinity of bacterial alpha-amylase for low grade

dextrins. This phenomenon may cause excessive starch breakdown into dextrins and result in sticky bread crumb characteristics.

Certain ions are known to exert a marked influence on amylase stability. Greater thermostability may cause excessive starch degradation with resulting sticky and gummy characteristics. Kneen, Sandstedt and Hollenbeck (15) have studied the stabilizing effect of calcium-ion on alpha-amylase extracts. Since the average patent flour contains approximately 0.018 percent calcium expressed on the dry weight basis (3), and considerable calcium is added to the bread formula in the form of dry milk solids, sufficient calcium may be present to lend greater thermostability to the alpha-amylase supplement.

Another problem arising from the use of certain supplements has been associated with the presence of excessive proteolytic enzyme activity. Since the work of Ford and Guthrie (5) on the proteinase of wheat flour, a number of theories and arguments have appeared in the literature on the role of proteinases in baking. Several reviews of the literature, Hildebrand (8), Hildebrand and Burkert (9), Miller and Johnson (18), Read and Haas (22), and Sandstedt and Fortman (23), on this subject have appeared.

Miller and Johnson (18) studied proteolysis in straight and sponge doughs. These workers (18) found that malt supplements containing excessive proteolytic activity produced inferior bread by the sponge procedure, but no detrimental effects were observed in the straight dough procedure. These differences were attributed to the partial inactivation of the proteinases by the sodium

chloride in the straight dough procedure. Furthermore, removal of part of the proteinase by absorption techniques resulted in a marked improvement in the bread when baked by the sponge dough procedure. Dirks and Miller (4) working with mold bran extracts were able to inactivate or remove 80 percent of the proteolytic activity by treatment with sodium chloride and pH adjustment, while removing only 10 percent of the alpha-amylase activity. Miller and Johnson (19) developed technics appropriate for the differential inactivation of alpha-amylase and proteinase in malted wheat and barley flour and fungal preparations.

Read and Haas (21) studied the effects of various sources of proteinase on bucky doughs. Considerable improvement in loaf volume, and grain as well as a reduction in the number of holes were obtained by addition of small amounts of several of the proteolytic products. The improvement was particularly true in sponge mixes. This was attributed to a mellowing action on the gluten, giving a more workable dough. Bucky doughs were greatly benefited by small amounts of certain plant proteinases. Excessive dosages of proteinase or "proteinase activator" ruined the baking properties of the gluten. The results indicated that certain flours have sufficient proteinase if the proteinase is activated.

The objective of this investigation was to study the characteristics of commercial bacterial and fungal alpha-amylase preparations and to determine the feasibility of their commercial use as alpha-amylase supplements. Consideration also was given

to means of alleviating the undesirable characteristics of certain preparations and to the retention of alpha-amylolytic and proteolytic activity in flours supplemented with commercial preparations and stored under controlled conditions.

MATERIALS AND METHODS

Materials

The major flour selected for this investigation was a commercial hard red winter, straight grade, unmalted sample having a protein content of 11.8 percent and an ash content of 0.45 percent. This flour showed good malt response. In addition, three hard red spring, unmalted baker's patent flours and two hard red winter, unmalted baker's patent flours ranging in protein content from 11.5 to 12.5 percent were used in studying the separate effect of alpha-amylase and proteinase supplementation.

The sources of the alpha-amylase supplements employed included a commercial malted wheat flour having an activity of 40 alpha-amylase units and eight commercial enzyme preparations. Five of the preparations, (Maltase-20, Diastase-29, Diastase-32, Diastase-33 and Diastase-34) were fungal diastase preparations. The other three preparations (Rhozyme-DX, Diastase-28 and Diastase-30) were bacterial diastase preparations.

Baking Procedures

Experimental Baking Procedure. The sponge dough procedure was employed for all baking studies. The sponges were mixed two

minutes in a "Hobart A-200"[1] mixer and fermented for four hours in a "Humi-Temp"[2] fermentation cabinet at 86°F. and 85 percent relative humidity. Remixing was continued with the Hobart mixer to the point of optimum development. After thirty minutes "floor time" the doughs were scaled to twenty ounces, and given twenty minutes rest before moulding with a "Century" moulder.[3] The loaves were proofed for 55 minutes at 92°F. and 85 percent humidity in an "Anets"[4] cabinet proof box. Baking was for 35 minutes in a "Reed" Reel Oven at 425°C.

A total of 700 grams of flour was used for each mix. The absorption was adjusted to the requirements of the flour. The following formula was used in the experimental baking procedure:

Ingredient	Sponge %	Dough %
Flour	70	30
Yeast	2	
Yeast food (Arkady[5])	0.5	
Sugar		5
Salt		2
Dry milk solids		3
Shortening		3
Dough conditioner (Paniplus[6])		0.5
Alpha-amylase	According to experiment	
Proteinase	According to experiment	
Water	70 percent of total	30 percent of total

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1 Manufactured by Hobart Manufacturing Co., Troy, Ohio.
2 Manufactured by the Research Products Co., Kansas City, Missouri.
3 Manufactured by Century Machine Co., Cincinnati, Ohio.
4 Manufactured by Bakers Engineering and Equipment Co., Kansas City, Mo.
5 Manufactured by Standard Brands Inc., Topeka, Kansas.
6 Manufactured by The Paniplus Company, Kansas City, Mo.

In a few cases a total of 300 grams of flour was used in each mix. With the following exceptions the procedure and formula were the same as for the larger doughs. The doughs were scaled to 250 grams and moulded by hand after punching with a "National" Sheeting Roll[1]. Proofing was completed in the "Humi-Temp" cabinet at 86°F. and 85 percent relative humidity for 55 minutes. The loaves were baked 25 minutes at 425°F. in the "Reed" Reel Oven.

Pilot Plant Bakery Procedure. The sponges were mixed five minutes in a "Day"[2] horizontal mixer, fermented at 86°F. and 85 percent relative humidity for four hours in the fermentation room[3] and remixed to optimum development as determined by the characteristics of the dough. After 30 minutes "floor time" the doughs were divided by hand, scaled to 20 ounces, and allowed to rest 20 minutes in a drawer proof cabinet. The doughs were molded in the "Century" moulder and proofed 55 minutes in the "Anets" cabinet at 92°F. and 85 percent relative humidity. Baking was completed in 35 minutes at 425°F. with the "Reed" Reel Oven.

The following formula was used in the pilot plant bakery investigations:

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1 Manufactured by National Manufacturing Co., Lincoln, Nebraska.
2 Manufactured by the J. H. Day Co., Cincinnati, Ohio.
3 Manufactured by Union Steel Products Co., Albion, Michigan.

Ingredient	Sponge	Dough
Flour	9 lbs.	6 lbs.
Yeast	4.8 oz.	
Yeast food (Arkady)	1.2 oz.	
Sugar		12 oz.
Salt		4.8 oz.
Dry milk solids		7.2 oz.
Shortening		4.8 oz.
Dough conditioner (Paniplus)		1.2 oz.
Water	60 percent of total	40 percent of total
Alpha-amylase	According to experiment	
Proteinase	According to experiment	

Designation of Alpha-amylase and Proteinase Concentration. Arbitrary terms were defined to indicate the proteinase and alpha-amylase concentrations used in baking. For alpha-amylase concentration the term "1X" indicated supplementation to an alpha-amylase level equivalent to the alpha-amylase added by 0.25 percent malted wheat flour. The term "1Y" indicated proteinase supplementation to the level that would be obtained by the addition to 100 grams of flour of that amount of proteinase which would give a delta titration of one ml in the proteolytic activity determination (17).

Activity of Enzyme Preparations

Alpha-amylase Activity. The preparations were extracted with 0.2 percent calcium chloride solution for one hour at 30°C.

and aliquots of the filtrate used for analysis. Alpha-amylase dextrinization activity was determined by the Wohlgemuth procedure as described by Sandstedt, Kneen and Blish (24). Values for alpha-amylase activity were expressed as the time in minutes required to produce the standard red-brown end point with iodine. These values are inversely proportional to the alpha-amylase activity.

Starch liquifying activity of the alpha-amylase was determined with the amylograph employing 65 grams of flour and 450 ml of liquid as described by Anker and Geddes (2).

Protinase Activity. Aliquots from extracts of the preparations were analyzed for proteolytic activity by the Ayre-Anderson method as modified by Miller (17). The procedure involves the measurement of non-protein nitrogen released during a five hour digestion of a bacto-hemoglobin substrate at controlled temperature and pH conditions. The delta titration in ml of 0.0714 N sodium hydroxide was used as the indication of proteolytic activity.

Thermostability of Alpha-amylases

The effect of temperature on the inactivation of the various alpha-amylases was determined by the technic used by Johnson and Miller (12). This involved heating the buffered enzyme solutions in the amylograph and the removal of aliquots after each five degree rise in temperature. The alpha-amylase activity of each liquot was determined and the results reported as the percent of the original activity remaining.

Differential Inactivation of Alpha-amylase and Proteinase

Technics appropriate for the differential inactivation of alpha-amylase and proteinase in Rhozyme-S were described by Dirks and Miller (4) and modified by Miller and Johnson (19). To inactivate the proteinase in Rhozyme-S the preparation was suspended in 0.2 percent calcium chloride solution (4 mg per ml) and adjusted while stirring rapidly to pH 10.5 with 2N sodium hydroxide. The resulting solution was heated at 50°C. for 30 minutes, cooled to room temperature and the pH adjusted to 6.0. This procedure resulted in retention of from 60 to 75 percent of the alpha-amylase activity while up to 98 percent of the proteinase was inactivated.

To inactivate the alpha-amylase in Rhozyme-S the preparation was suspended in water (4 mg per ml) and the pH adjusted to 4.0 with 2N sulfuric acid. After heating at 50°C. for 30 minutes, the solution was cooled to room temperature and the pH adjusted to 6.0. Approximately 70 percent of the proteinase was retained while only 10 percent of the alpha-amylase activity remained.

Effect of Storage on Enzyme Activity

To study the effects of storage on enzyme activity, flour was supplemented with Rhozyme-S to the alpha-amylase equivalent of 1 percent malted wheat flour. Alpha-amylase and proteinase activity were determined at bimonthly intervals. Possible acceleration of storage deterioration by the presence of oxygen was

studied by storing the supplemented flour for eight months in jars containing oxygen, nitrogen and air atmospheres at 35°C., cold room (5°C. to 9°C.), and room (20°C. to 40°C.) temperatures. The gaseous atmospheres were obtained in the following manner. Copper tubes were soldered into lids of half gallon jars, a short piece of rubber tubing attached and a screw clamp applied to give an air tight seal. After the supplemented flour was placed in the jars, the jar was alternately exhausted of air and filled with oxygen or nitrogen to a pressure measured by an arbitrary height of mercury. This procedure was repeated three times to insure replacement of the air by the gas. At the end of the third cycle the pressure was reduced to atmospheric pressure. The gases were replenished at bimonthly intervals.

Compressibility Measurements of Bread

A "Bloom"[1] gelometer was used to study the effect of alpha-amylase and proteinase supplementation on bread crumb compressibility. The experimental value recorded was the weight of lead shot required to press a one-inch plunger 4 mm. into a slice of bread. Two determinations on each of two slices cut from three loaves chosen at random from each experimental group were recorded.

Bacterial Spore Count Determination

The official A.A.C.C. method (1) for determining the total

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1 Manufactured by Precision Scientific Co., Chicago, Illinois.

bacterial spores in flour was followed in making the spore counts of the various prep rati s.

EXPERIMENTAL RESU TS AND DISCUSSION

Alpha-amylase and rotinase Activity

Eight commercial fungal and bacterial enzyme concentrates were analyzed for alpha-amylase and proteinase and compared to the respective enzyme activities of commercial malted wheat flour. Rhozyme-5 contained over one hundred times the alpha-amylase activity of malted wheat flour (Table I). This concentrate also possessed very high proteolytic activity resulting in a proteinase -- alpha-amylase activity ratio over eight times as great as that for malted wheat flour. Diastase-29 was observed to be low in alpha-amylase activity but high in proteolytic activity. Diastase-33, -34, and -28 were found to have low proteinase -- alpha-amylase activity ratios.

Amylase supplements used in baking are normally standardized on dextrinogenic activity (14). Since Rhozyme-5 is standardized by the manufacturer on saccharifying activity, it was desirable to check the dextrinogenic activity in a series of samples from commercial batches. An analysis of variance of the alpha-amylase activity for these samples (Table 2) showed that the differences were highly significant. This suggests that the enzyme concentrates should be standardized on dextrinogenic activity.

Table I. Alpha-amylase and proteolytic activities of the various preparations compared to malted wheat flour.

Enzyme	Ratios of activities		
	Alpha-amylase	Proteinase	Ratio of proteinase to alpha-amylase
Malted wheat flour	1	1	1
Maltase-20 (first sample)	74	140	1.9
Maltase-20 (large sample)	66	67	1+
Rhozyme-8	120	1000	8.3
Diastase-29	0.21	417	2000
Diastase-32	5.7	244	43
Diastase-33	50	31.6	.63
Diastase-34	80	0.19	.002
Diastase-28	31	5.7	.18
Diastase-30	24	69	2.9

Table 2. Data and analysis of variance of alpha-amylase activities in a series of Rhozyme-S samples.

Sample number	T[1] 1st aliquot[2]	T[1] 2nd aliquot[2]
	Min.	Min.
241	10.12	10.25
249	9.75	9.75
251	8.88	9.37
260	11.25	11.75
262	10.00	10.37

Analysis of variance

Source of variation	Degrees of freedom	Mean square	F ratio
Individual determinations	5	0.0642	
Samples	4	1.5180	23.64[3]
Total	9		

1 T = time in minutes required to reach the standard brown end point.
2 Each aliquot equivalent to 2 mg of preparation.
3 Significant at the 1 percent level.

Preliminary Experimental Baking

An experimental baking study using extracts of Rhozyme-S, Maltase-20 (1st sample), Diastase-28, -29, -30, and malted wheat flour was made to determine the general baking characteristics of the various preparations (Table 3). Diastase-29 caused bread to have low volume, open grain and poor texture. Diastase-28 and Diastase-30 produced bread with large volume, and sticky and gummy crumb. Bread produced with Maltase-20 and Rhozyme-S was

Table 3. Comparison of Rhozyme-S, Maltase-20, Diastase-28, -29, and -30 with malted wheat flour as amylase supplements in baking.

Enzyme	Concentration[1]	External characteristics	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
--	0	Poor	70	65	805	OK
Malted wheat flour	1X	Good	90	90	970	OK
"	4X	Good	85	85	1000	OK
"	8X	Good plus	80	80	1015	Slightly sticky
Rhozyme-S	1X	Fair (rough)	90	92	915	OK
"	4X	Fair (rough)	85	90	960	Slightly sticky
"	8X	Very good	80	85	980	Sticky
Maltase-20	1X	Good	80	85	985	OK
"	4X	Good	75	80	1025	OK
"	8X	Good (rough)	75	75	980	Slightly sticky
Diastase-28	1X	Fair	75	60[2]	980	OK
"	4X	Very good	70	50[2]	1010	OK
"	8X	Good	65	40[2]	985	Slightly sticky
Diastase-29	.001X	Fair (rough)	70	65	910	Sticky
"	.004X	Fair	70	65	925	Sticky
"	.008X	Poor	70	65	935	Very sticky
Diastase-30	1X	Good	80	60[2]	985	OK
"	4X	Good	60	50[2]	1025	OK
"	8X	Very good	55	40[2]	1020	Slightly Sticky

1 IX = concentration equivalent to the alpha-amylase provided by .25 percent malted wheat flour.
2 Sticky crumb characteristics.

slightly inferior but comparable in volume, grain and texture to that baked with malted wheat flour.

Experimental baking results obtained with Diastase-32, -33, and -34 are recorded in Table 4. Diastase-32 was similar to Diastase-29, in that sufficient proteinase was present to produce excessive gluten breakdown before the beneficial effects of the alpha-amylase appeared. Bread produced with Diastase-33 and -34 was quite comparable to that obtained with malted wheat flour. There was no tendency for doughs containing Diastase-34 to slacken excessively during fermentation. A slight slackening was observed, however, in the dough to which 3X concentration of Diastase-33 was added. Thus, satisfactory bread was produced with Rhozyme-S, Maltase-20, Diastase-33, and Diastase-34. Diastase-29 and -32 caused the sponges to liquify before beneficial alpha-amylase effects were obtained. Bread produced with the two bacterial preparations, Diastase-28 and -30, was unsatisfactory because of sticky bread crumb characteristics.

Investigation of Diastase-28 and -30

Thermostability of Alpha-amylases. The effect of temperature on the inactivation of alpha-amylases was studied as one of the possible causes of crumb stickiness resulting from the use of Diastases-28 and -30. The data recorded in Table 5 indicated that the bacterial alpha-amylases, Diastase-28 and -30, were less thermostable than malted wheat flour alpha-amylase. These results were not in agreement with the work of Johnson and Miller (12). The investigation was repeated including a bacterial

Table 4. Comparison of Diastase-32, -33 and -34 and malted wheat flour as amylase supplements in baking.

Enzyme	Concentration[1]	External characteristics	Grain	Texture	Loaf Volume	Dough properties
			%	%	ml	
Malted wheat flour	1X	Fair	87	90	3125	OK
"	4X	Good	85	85	3475	OK
"	8X	"	88	85	3365	Slightly slack
Diastase-32	1X	Very poor	60	60	2775	Almost liquefied
"	4X					Liquefied
"	8X					Liquefied
Diastase-33	1X	Good to very good	87	88	3190	OK
"	4X	" " " "	85	86	3225	OK
"	8X	Very good	83	84	3240	Slightly slack
Diastase-34	1X	Good	85	85	3115	OK
"	4X	"	85	85	3240	OK
"	8X	Good plus	83	83	3375	OK
"	0	" "	75	80	2900	OK

1 IX = concentration equivalent to alpha-amylase provided by .25 percent malted wheat flour.

Table 5. Percentages of alpha-amylase activity remaining after heating to different temperatures in suspending medium containing 0.2 percent calcium chloride.

Temperature	Rhozyme-S	Malted Wheat flour	Diastase-28	Diastase-30
°C	%	%	%	%
85	0	0	0	0
80	0	5	0	0
75	6	58	3	11
70	69	94	39	74
65	100	98	96	93
60	100	100	100	100

preparation (Rhozyme-DX) found by these workers to possess high thermostability. As an added precaution the 0.2 percent calcium chloride was omitted from the suspending medium since the manufacturer had removed calcium from the bacterial preparations in an attempt to lower their thermostabilities. The results (Plate I) show that the alpha-amylases of Diastase-28 and -30 were less thermostable than malted wheat flour alpha-amylase. Rhozyme-DX, as expected, possessed high thermostability. These results corroborate the work of Tilden and Hudson (26) showing that alpha-amylases from different bacterial strains may differ in thermostability.

Alpha-amylase Activities at Higher Temperatures. The effect of higher temperatures on relative alpha-amylase activities from various sources was investigated. The percentage increases in

EXPLANATION OF PLATE I

The effect of heating and enzyme source on the retention of alpha-amylase activity.

Curve a. Diastase-28

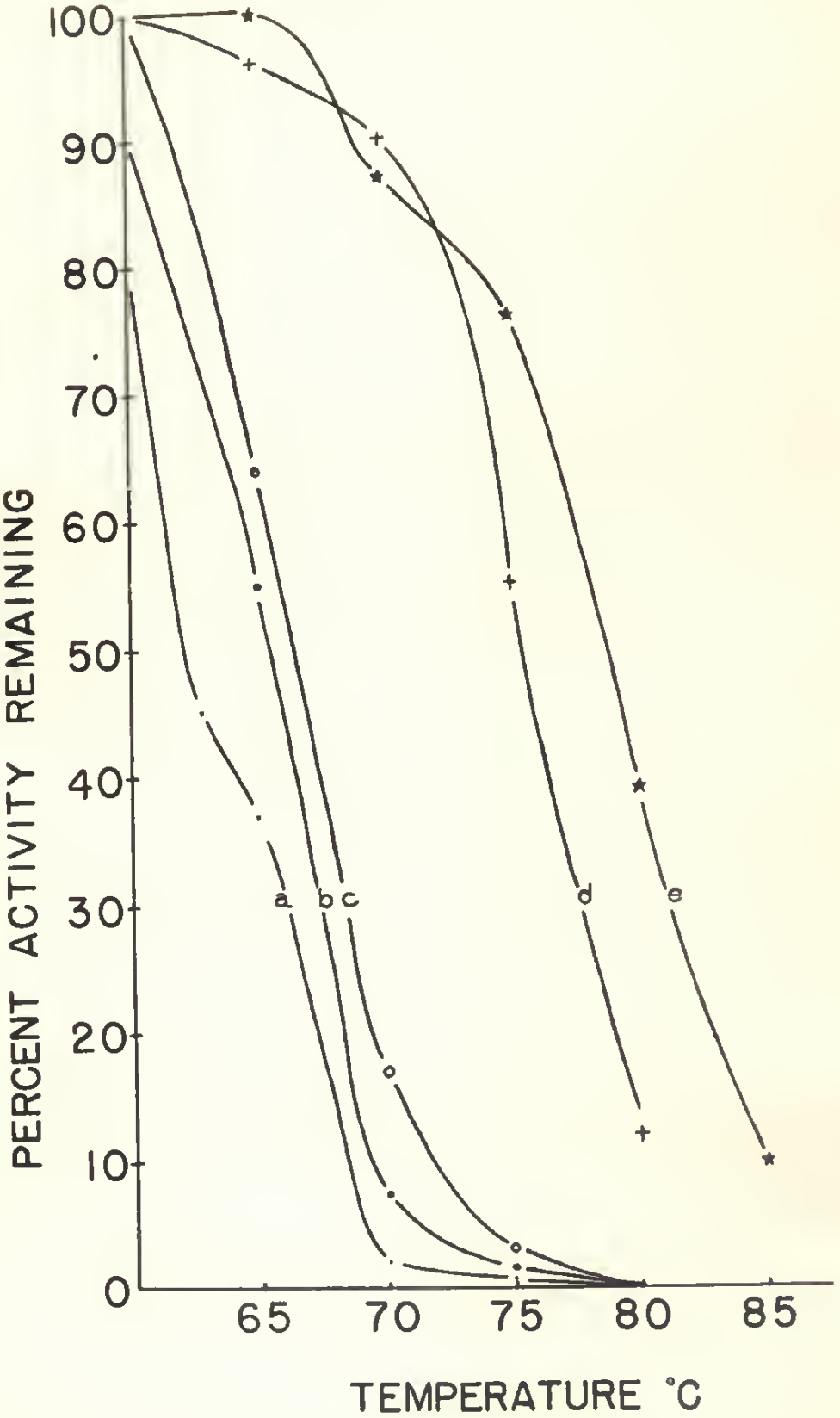
Curve b. Diastase-30

Curve c. Rhozyme-S

Curve d. Malted wheat flour

Curve e. Rhozyme-DX

PLATE I


100
90
80
70
60
50
40
30
20
10
0
PERCENT ACTIVITY REMAINING
65
70
75
80
85
TEMPERATURE °C
a
b
c
d
e

activity at 50°C. as compared with the activity at 30°C. are recorded in Table 6. Increasing the temperature to 50°C. caused the alpha-amylase of malted wheat flour to increase in activity more than the alpha-amylases of Diastase-28 or -30. This suggests that at the temperatures of baking, the alpha-amylase activities of Diastase-28 and -30 increase less than the activity of malted wheat flour alpha-amylase.

Amylograph Curve Heights. The effect of various sources of alpha-amylase on the maximum viscosity of a starch paste as measured with the amylograph was investigated. Equivalent amounts of the various alpha-amylases in the absence of calcium chloride as a stabilizer were employed. Maximum viscosity values (Table 7) lower than the maximum malted wheat flour viscosity values were obtained when equivalent amounts of Diastase-28 and -30 (based on dextrinogenic activity at 30°C.) were used. Thus, equivalent amounts of Diastase-28 and -30 would apparently produce greater starch degradation than an equivalent amount of malted wheat flour.

From the inactivation data and dextrinization activities at higher temperature, malted wheat flour might be expected to produce greater starch degradation with resulting lower maximum viscosities and possibly sticky bread crumb. This apparent anomaly may be explained by assuming a lesser affinity of bacterial alpha-amylase for lower molecular weight dextrins. Accordingly bacterial alpha-amylase molecules may be free to split greater numbers of starch molecules, with a corresponding increase in dextrin formation and stickiness of bread crumb.

Table 6. Effect of calcium chloride and increased temperature on activity of various alpha-amylases.

Preparation	In $CaCl_2$ extract	In water extract
	%[1]	%[1]
Malted wheat flour	234	---
Diastase-28	---	129
Diastase-30	166	164
Maltase-20	127	104
Diastase-29	137	110

1 Percentage increase due to increase in temperature from 30°C. to 50°C.

Table 7. Effect of alpha-amylases from various sources on maximum amylograph curve heights.

Preparation	Concentration[1]	Maximum height	Percent height of corresponding malted wheat flour curve
		(B.U.)[2]	%
Malted wheat flour	1X	385	--
" " "	½X	630	--
Diastase-28	1X	340	88
" "	½X	550	87
Diastase-30	1X	280	73
" "	½X	480	76

1 1X Concentration equivalent to the alpha-amylase provided by 25 percent malted wheat flour supplementation.
2 1X Brabender Units.

Experimental Baking. Experimental baking using Diastase-28 and -30 at 0.25X, 1X, and 4X concentrations again indicated that these two bacterial preparations cause sticky crumb characteristics. A slight improvement in crumb characteristics for both sources was observed when calcium was omitted. The bread crumb containing 0.25X concentration of Diastase-28 was least sticky, but when tasted stuck to the teeth and roof of the mouth resulting in an unpleasant sensation.

An investigation was made to determine if an excess of saccharifying amylase would remove the sticky characteristics. The loaves containing 1X concentrations of Diastase-28 or -30 in the presence of as much as 4X concentration of Rhozyme-S (saccharifying enzyme source) were found to possess very sticky and gummy bread crumbs. These results indicate that the added saccharifying enzyme was probably inactivated in the oven before the starch liquifying enzyme was inactivated.

Another experimental baking study was completed to determine the concentration at which the crumb stickiness produced by Diastase-28 and -30 became evident. Stickiness was evident at 0.1X concentration of either preparation, and was very noticeable when 0.25X concentration of either enzyme was used (Table 8). Stickiness was not observed at 0.04X concentration in either case. However, the grain and texture scores for these loaves were slightly lower than for the control loaves. No increase in volume was obtained with 0.1X concentration of Diastase-28 while 0.1X Diastase-30 caused a slight increase in volume. From these results it was concluded that as the concentration of Diastase-28

Table 8. Effect of starch liquifying and saccharifying enzymes on development of crumb stickiness.

Rhozyme-S[1] concentration	Diastase-28[1] concentration	External characteristics	Grain	Texture	Loaf volume
			%	%	ml.
0	0.04X	Fair	68	70	2835
0	0.1 X	"	65	65[2]	2840
0	0.25X	"	67	65[3]	2880
1X	1X	Good	50	50[4]	3015
4X	1X	"	55	50[4]	2940
	Diastase-30 concentration				
0	0.04X	Fair	65	65	2950
0	0.1 X	"	65	65[2]	3060
0	0.25X	Fair to good	60	65[3]	2900
1X	1X	Good	55	50[4]	3025
4X	1X	"	50	50[4]	2915
1X	0	"	85	80	2975
4X	0	Fair to good	80	83	2925
0	0	Poor	70	75	2865

[1] 1X = concentration of alpha-amylase equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.
[2] Slightly sticky bread crumb.
[3] Sticky bread crumb.
[4] Very sticky bread crumb.

or -30 was increased sticky bread crumb resulted before any beneficial effects appeared. Thus, Diastase-28 and -30 could not be used as alpha-amylase supplements for flour.

Investigation of Rhozyme-S and Maltase-20

Optimum Supplementation. An investigation of optimum supplementation with Rhozyme-S and Maltase-20 (first sample) using concentrations of 1X, 4X and 8X was made in the pilot plant bakery (Table 9). Although the doughs slackened slightly, the dough handling characteristics were satisfactory for doughs containing 1X concentration of either Rhozyme-S or Maltase-20. The doughs containing 4X concentration of Rhozyme-S and 8X concentration of Maltase-20 slackened considerably. The doughs containing 8X concentration of Rhozyme-S slackened sufficiently to make dough handling difficult. The most desirable grain and external characteristics were observed when 1X concentration of either Rhozyme-S or Maltase-20 was used. The texture of the 1X and 4X Rhozyme-S supplemented loaves was approximately the same, while deterioration was observed at 8X concentration. The optimum texture was observed at 1X concentration of Maltase-20 and progressive deterioration appeared when additional amounts were used. Optimum results were obtained when 1X concentration of either Rhozyme-S or Maltase-20 were used.

Dilution of Rhozyme-S and Maltase-20 Preparations. Rhozyme-S and Maltase-20 (2nd sample) were blended with flour to produce resulting mixtures with alpha-amylase activities equivalent to the alpha-amylase activity of commercial malted wheat flour.

Table 9. Pilot plant baking data indicating the optimum supplementation levels of Rhozyme-S and Maltase-20 for a hard red winter flour.

Preparation	Relative[1] concentration	External characteristics	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
---	0	Good	80	75	2925	OK
Rhozyme-S	1X	Good to very good	85	85	3025	OK
"	4X	Fair to good	75-o	85	3025	OK
"	8X	Good	70-o	80	3145	Slack
Maltase-20	1X	Very good	85	87	3040	OK
"	4X	Good	75-o	82	3070	OK
"	8X	Good	70-o	75	2975	Slightly slack

1 1X = concentration equivalent to the alpha-amylase provided by .25 percent malted flour supplementation.

These blends were studied as alpha-amylase supplements in the pilot plant bakery.

The results (Table 10) were essentially the same as those obtained when extracts of the preparations were used. Again the greater proteolytic activity of Rhozyme-S was evidenced by the slackening of the dough containing 4X concentration of Rhozyme-S. Therefore, if such enzyme preparations were used without proper control, the baker could use too much enzyme and experience serious trouble with gluten breakdown. The dough to which 4X Maltase-20 was added slackened only slightly. The bread produced using Maltase-20 graded slightly higher than the bread produced with Rhozyme-S. Bread baked with Rhozyme-S and Maltase-20 supplementation was comparable to that produced with malted wheat flour.

The results showed that it was feasible to dilute commercial enzyme concentrates with flour or other suitable materials to the alpha-amylase activity of commercial malted wheat flour. Thus, malt feeders now in use in flour mills could be used to blend in the diluted amylase preparations.

Partial Removal of Rhozyme-S Proteinase. Repeated baking investigations indicated that the proteinase activity of Rhozyme-S was too high. Accordingly, solutions of Rhozyme-S having 25 percent, 50 percent, 75 percent, and 90 percent of the proteinase inactivated were studied in the pilot plant bakery employing 1X and 4X concentrations of the supplement. The doughs containing 4X concentration of alpha-amylase slackened somewhat

Table 10. The effect of using Rhozyme-S and Maltase-20, diluted with flour to an alpha-amylase activity equivalent to malted wheat flour, as alpha-amylase supplements.

Preparation	concentration[1]	External characteristics	Grain	Texture	volume	Dough properties
			%	%	ml	
---	0	Fair	80	80	2858	OK
Malted wheat flour	1X	Good	90	90	2971	OK
" " "	4X	Good plus	92	90	3025	Slightly slack
Rhozyme-S	1X	" "	87	85	2992	Slightly slack
" "	4X	Good	80	83	2963	Slack
Maltase-20	1X	Very good	90	88	2942	OK
" "	4X	Good	88	88	3058	Slightly slack

1 1X = concentration equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.

when 50 percent of the proteinase was retained. Considerable slackening was observed at 4X concentration when 75 percent of the proteinase was retained. The best bread was obtained when only 10 percent to 25 percent of the proteinase was retained (Table 11). Thus, the desirability of reducing the proteinase activity of Rhozyme-S was demonstrated.

Separate Alpha-amylase and Proteinase Supplementation

Requirements of Hard Red Winter Wheat Flour. The separate effects of alpha-amylase and proteinase supplementation were studied in the pilot plant bakery. Rhozyme-S with up to 98 percent of the proteinase inactivated was used in sufficient amounts to provide 0, 1X, 4X, and 8X alpha-amylase supplementation. In combination with this alpha-amylase, Rhozyme-S proteinase was added in amounts equivalent to the proteinase activity of 0, 0.5X, 1X, and 4X concentrations of untreated Rhozyme-S. Both external and internal characteristics, (Tables 12 and 13) indicated that 4X to 8X concentration of Rhozyme-S with the proteinase inactivated provided optimum alpha-amylase supplementation for the major hard red winter flour. Some improvement in the quality of the bread was observed for proteinase supplementation up to that concentration equivalent to the proteinase in 1X concentration of Rhozyme-S.

Since considerable alpha-amylase was not inactivated in securing a source of proteinase from Rhozyme-S, the utilization of Diastase-29 as a source of proteinase was investigated.

Table 11. Pilot plant bakery baking data indicating the effect on baking with Rhozyme-3 having 25 percent, 50 percent, 75 percent and 90 percent of the proteinase removed.

Alpha-amylase[1] concentration	Proteinase retained	External characteristics	Grain	Texture	Loaf volume	Dough properties
	%		%	%	ml	
0	--	Fair	70	70	2833	OK
1X	10	Very good	85	90	2916	OK
1X	25	Good	75	85	2796	OK
1X	50	"	80	80	2696	Slightly slack
1X	75	Very good	75	75	2879	Slack
4X	10	" "	85	90	3008	OK
4X	25	" "	83	88	2971	OK
4X	50	Good plus	78	85	2946	Slack
4X	75	Fair plus (rough)	75	75	2879	Slack

1 1X = Concentration equivalent to the alpha-amylase provided by 25 percent malted wheat flour.

Table 12. Pilot plant baking data indicating the separate effect of alpha-amylase and proteinase supplementation.

Rhozyme-S[1] alpha-amylase concentration	Rhozyme-S[2] proteinase concentration	External characteristics	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
0	0	Fair plus	70	75	2925	OK
1X	0	Good plus	70	80	2925	OK
4X	0	Good	75	85	2985	OK
8X	0	Fair plus	80	85	2903	OK
0	1X	Good	88	88	2972	OK
0	4X	Fair plus	85	85	3060	Slightly slack
1X	1X	Good	90	90	2978	OK
4X	4X	Very good	88	88	2978	Slightly slack

1 1X = concentration equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.
2 1X = concentration of proteinase equivalent to the proteinase present in 1X alpha-amylase concentration of Rhozyme-S.

Table 13. Pilot plant baking data indicating the separate effect of Rhozyme-S proteinase and alpha-amylase supplementation.

Alpha-amylase[1] concentration	Proteinase[2] concentration	External characteristics	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
1X	½X	Good	80	80	2950	OK
1X	1X	Good	85	85	2930	OK
1X	4X	Good plus	75	80	3055	Slightly slack
3½X	½X	Good	90	90	3065	OK
4X	1X	Good plus	75	80	3070	Very slack[3]
4X	4X	Very good	90	90	3120	Slightly slack
8X	½X	Good	75	75	3015	OK
8X	1X	Good plus	70	70	3130	Slightly slack

1 1X = concentration of alpha-amylase equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.
2 1X = concentration of proteinase equivalent to the proteinase in 1X concentration of untreated Rhozyme-S.
3 Too much water was added by mistake causing excessive slackness.

Rhozyme-S from which the proteinase had been removed was used as the source of alpha-amylase. Experimental pound loaves using 1X, 4X and 8X concentrations of alpha-amylase in combination with 0.5Y, 1Y and 4Y concentrations of Diastase-29 were baked. Diastase-29 supplementation up to 0.5Y proteinase concentration caused no marked improvement or deterioration in the quality of the bread baked with the major hard red winter flour, however, 1Y and 4Y concentrations produced progressively inferior bread as well as excessive softening of the doughs. The 4X Rhozyme-S alpha-amylase combined with 0.5Y Diastase-29 proteinase produced grain, texture, and external characteristics (Table 14) comparable to those produced by 1X and 4X concentrations of malted wheat flour. The results indicated that Diastase-29 was a satisfactory source of proteinase for use in further investigations.

In additional experimental baking investigations 0, 0.5Y, 1Y and 4Y concentrations of Diastase-29 were used in combination with 0, 1X, 4X, and 8X concentrations of Rhozyme-S alpha-amylase. Based on grain and texture scores, 0 to 0.5Y proteinase supplementation was required to secure the optimum quality bread from hard red winter flour No. 2 (Table 15). A third hard red winter flour also required from 0 to 0.5Y concentration of Diastase-29 to produce the optimum bread. The level of alpha-amylase supplementation required by these flours did not appear to be critical.

Supplementation with diastase-29 at 0, 1Y, and 4Y concentrations in combination with 0, 1X, and 8X concentrations of

Table 14. Experimental baking data using Rhozyme-S alpha-amylase and Diastase-29 proteinase to supplement a hard red winter wheat.

Alpha-amylase concentration[1]	Diastase-29 proteinase concentration[2]	External characteristics[1]	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
0	0	Poor	65	70	2990	OK
1X	½Y	Good	90	85	3040	OK
1X	1Y	Good	85	85	3100	Slightly slack
1X	4Y	Good	80	80	3100	Slack
4X	½Y	Good	90	90	3225	OK
4X	1Y	Good	85	85	3150	Slightly slack
4X	4Y	Good plus	80	80	3165	Slack
8X	½Y	Good plus	90	85	3150	OK
8X	1Y	Good plus	85	85	3250	Slack
6X	4Y	Very good	85	80	3065	Slack
Rhozyme-S concentration						
1X		Fair	85	90	3285	OK
4X		Good	90	85	3115	slightly slack
8X		Good	90	80	3125	Slack
Malted wheat flour concentration						
1X		Good plus	90	90	3140	
4X		Good	90	90	3225	
8X		Good	85	85	3300	Slightly slack

1 1X = concentration of alpha-amylase equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.
2 1Y = the addition to 100 grams of flour that amount of Diastase-29 proteinase which would give a delta titration of one ml in a proteolytic activity determination.

Table 15. The separate effect of alpha-amylase and proteinase supplementation on hard red winter wheat flour No. 2.

Rhozyme-S[1] alpha-amylase concentration	Diastase-29[2] concentration	External characteristics	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
0	0	Poor	85	80	3025	OK
0	½Y	Fair	85	85	3000	OK
0	1Y	Fair	80	80	3100	OK
0	4Y	Good	85	80	3150	Slack
1X	0	Good	90	90	3065	OK
1X	½Y	Good to very good	88	88	3115	OK
1X	1Y	Good	85	85	3015	OK
1X	4Y	Good	85	83	3000	Slack
4X	0	Good	90	88	3040	OK
4X	½Y	Good to very good	90	88	3075	OK
4X	1Y	Good	85	83	3100	Slightly slack
4X	4Y	Good	85	83	3140	Slack
8X	0	Good to very good	85	85	2850	OK
8X	½Y	" " " "	85	85	2865	Ok
8X	1Y	Good	80	83	3025	Slightly slack
8X	4Y	Good	80	83	3050	Slack
Malted wheat flour concentration						
1X		Good	90	92	3040	OK
4X		Good to very good	88	88	3100	OK
8X		" " " "	90	88	3215	Slightly slack

1 1X = concentration of alpha-amylase equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.
2 1Y = the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a delta titration of one ml in a proteolytic activity determination.

Rhozyme-S alpha-amylase was used in pilot plant bakery investigations of hard red winter wheat flours No. 2 and No. 3. The optimum bread was obtained when no proteinase was added to flour No. 3 (Table 16), but the flour easily tolerated proteinase up to 1Y concentration. Flour No. 2 essentially confirmed these results with a tendency toward slightly more deterioration at 1Y concentration of Diastase-29.

From these results, it was concluded that the optimum quality bread was obtained when the hard red winter wheat flours received 0 to ½Y proteinase supplementation. The level of alpha-amylase supplementation up to 8X concentration did not appear to be critical.

Requirements of Hard Red Spring Wheat Flour. A composite of hard red spring wheat flours was experimentally baked with the usual combinations of proteinase and alpha-amylase supplementation. Excessive buckiness was not observed in the control doughs. Concentrations of 0.5Y and 1Y Diastase-29 caused some improvement in grain and texture (Table 17), while at 4Y concentration gluten breakdown was indicated by dough softening and by grain and texture deterioration. The optimum level of alpha-amylase supplementation was 4X concentration, however, this level was not critical. This experiment was repeated three times using different hard red spring flours. These flours gave the best results when supplemented with 0.5Y to 1Y concentration of Diastase-29 proteinase while the level of alpha-amylase supplementation was not critical.

The three hard red spring flours, supplemented with

Table 16. Pilot plant baking data indicating the separate effect of alpha-amylase and proteinase supplementation of hard red winter wheat flour No. 3.

Rhozyme-S[1] alpha-amylase concentration	Diastase-29[2] concentration	External characteristics	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
0	0	Good	75	80	2916	Good
0	1Y	Good	80	78	2781	Good
0	4Y	Good plus	85	80	2988	slightly slack
1X	0	Good plus	90	88	3059	Good
1X	1Y	Very good	90	88	3041	Slightly slack
1X	4Y	Good plus	88	85	2959	Slack
8X	0	Good	90	90	3062	Good
8X	1Y	Good	88	90	3066	Slightly slack
8X	4Y	Good plus	85	87	3056	Slack

1 1X = concentration of alpha-amylase equivalent to the Alpha-amylase provided by .25 percent malted wheat flour supplementation.

2 1Y = the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a delta titration of one ml in a proteolytic activity determination.

Table 17. Experimental baking data indicating the separate effect of alpha-amylase and proteinase supplementation on a composite hard red spring flour.

Rhozyme-S[1] alpha-amylase concentration	Diastase-29[2] concentration	External characteristics	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
0	0	Good	65	70	3290	OK
1X	0	Very good	75	75	3540	OK
1X	½Y	Very good	87	85	3450	OK
1X	1Y	Good	87	85	3425	OK
1X	4Y	Good	85	85	3400	Slightly slack
4X	0	Very good	75	75	3500	OK
4X	½Y	Very good	85	75	3565	OK
4X	1Y	Good	90	90	3425	OK
4X	4Y	Very good	90	85	3350	Slightly slack
8X	0	Very good	80	80	3540	OK
8X	½Y	Good	82	80	3440	OK
8X	1Y	Very good	82	83	3475	Slightly slack
8X	4Y	Very good	85	80	3437	Slack
0	½Y	Very good	83	80	3337	OK
0	1Y	Very good	85	83	3325	OK
0	4Y	Good	83	80	3375	Slack

1 1X = concentration of alpha-amylase equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.
2 1Y = the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a delta titration of one ml in a proteolytic activity determination.

Diastase-29 proteinase and Thermyme- alpha-amylase were baked in the pilot plant bakery. The optimum quality bread was obtained when 1Y to 4Y concentration of Diastase-29 proteinase was used (Table 18). The results with the other two hard red spring flours also suggested that at least 1Y concentration of Diastase-29 was required for optimum proteinase supplementation. Little difference appeared between 1X and 8X alpha-amylase supplementation.

These results obtained with hard red spring wheat flour indicated the optimum level of proteinase supplementation was substantially different from that required by hard red winter wheat flours. Hard red spring wheat flours in general require more proteinase than do hard red winter wheat flours.

Effect on Crumb Compressibility. The separate effect of alpha-amylase and proteinase supplementation on bread crumb compressibility was studied. The data are presented in Tables 19 and 20. The data in Table 19 were obtained 24 hours after baking, while the data in Table 20 were taken on duplicate loaves 94 hours after baking. An analysis of variance (Table 21) of these data showed that significant differences in the bread crumb compressibilities are caused by alpha-amylase, proteinase, and length of storage. Both alpha-amylase and proteinase increase the compressibility while storage decreases the compressibility.

Least significant mean differences were determined by the method of Snedecor (25). From these calculations it was concluded that the compressibility values for bread containing 4Y concentration of proteinase were significantly lower than the

Table 18. Pilot plant baking data indicating the separate effect of alpha-amylase and proteinase supplementation of hard red spring wheat flour.

Rhozyme-S alpha-amylase concentration	Diastase-29[2] concentration	External characteristics	Grain	Texture	Loaf volume	Dough properties
			%	%	ml	
0	0	Fair	75	80	2788	Slightly bucky
0	1Y	Good	77	82	2738	Good
0	4Y	Good	77	82	2869	Good
1X	0	Good	80	85	2747	Good
1X	1Y	Good	85	87	2975	Good
1X	4Y	Very good	92	90	2994	Slightly slack
8X	0	Good	80	87	2888	Good
8X	1Y	Very good	92	90	2953	Slightly slack
8X	4Y	Good plus	92	90	2959	Slightly slack

1 1X = concentration of alpha-amylase equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.
2 1Y = the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a delta titration of one ml in a proteolytic activity determination.

Table 19. Twenty four hour compressibility values for bread baked with different levels of alpha-amylase and proteinase.

Alpha-amylase[1] concentration	Proteinase concentration	Loaf No.	Compressibility values for two slices per loaf[3]				Group mean
			gms.	gms.	gms.	gms.	
0	0	3c	98	99	99	105	
"	"	3b	104	106	98	108	
"	"	2c	88	100	90	110	100.4
0	1Y	5c	108	105	97	100	
"	"	6b	96	103	94	87	
"	"	4b	83	80	98	90	95.1
0	4Y	7b	84	79	86	94	
"	"	8b	92	90	81	90	
"	"	9c	75	94	85	78	85.8
1X	0	11c	85	85	77	83	
"	"	11b	75	92	81	86	
"	"	12c	85	91	85	82	83.9
1X	1Y	15c	74	80	67	96	
"	"	15b	76	86	90	92	
"	"	14c	84	83	79	80	82.3
1X	4Y	17b	68	66	67	67	
"	"	16b	83	77	74	87	
"	"	17c	66	84	66	88	74.6
8X	0	21b	76	85	81	84	
"	"	21c	78	80	84	90	
"	"	19b	68	82	86	92	82.2
8X	1Y	22b	67	66	75	77	
"	"	24b	71	70	82	81	
"	"	24c	79	88	82	72	75.8
8X	4Y	26b	71	65	77	71	
"	"	25c	78	77	74	71	
"	"	27c	60	75	68	63	71.0

1 1X concentration of alpha-amylase equivalent to the alpha-amylase provided by 25 percent malted wheat flour supplementation.

2 1Y the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a delta titration of one ml in a proteolytic activity determination.

3 The grams of lead shot required to press a one inch plunger 4 mm. into the bread crumb.

Table 20. Ninety four hour compressibility values for bread baked with different levels of alpha-amylase and proteinase.

Alpha-amylase[1] Concentration	Proteinase[2] concentration	Loaf No.	Compressibility values for two slices per loaf[3]				Group mean
			gms.	gms.	gms.	gms.	gms.
0	0	1c	157	144	146	177	
"	"	1b	168	146	176	157	
"	"	2b	139	128	154	132	152.0
0	1Y	6c	130	129	108	124	
"	"	5b	165	131	169	171	
"	"	4c	188	166	149	162	149.3
0	4Y	7c	133	151	124	148	
"	"	9b	143	130	147	142	
"	"	8c	150	146	163	156	144.4
1X	0	12b	122	118	141	134	
"	"	10b	137	139	153	126	
"	"	10c	132	140	113	124	131.6
1X	1Y	14b	140	118	127	125	
"	"	13c	123	111	129	122	
"	"	13b	130	122	131	126	125.3
1X	4Y	17c	118	116	138	138	
"	"	17b	113	107	123	125	
"	"	18b	112	128	113	110	120.5
8X	0	20c	115	141	131	123	
"	"	20b	142	127	115	143	
"	"	19c	119	125	127	112	126.6
8X	1Y	22c	134	112	123	124	
"	"	23b	119	100	119	117	
"	"	23c	123	150	100	124	120.4
8X	4Y	25b	115	129	130	123	
"	"	26c	137	118	106	126	
"	"	27b	113	122	98	107	118.7

[1] 1X concentration of alpha-amylase equivalent to the alpha-amylase provided by .25 percent malted wheat flour supplementation.

[2] 1Y the addition to 100 grams of flour of that amount of Diastase-29 proteinase which would give a delta titration of one ml. in a proteolytic activity determination.

[3] The grams of lead shot required to press a one inch plunger 4 mm. into the bread crumb.

Table 21. Analysis of variance of the separate effect of alpha-amylase, proteinase, and storage on bread crumb compressibility.

Source of variation	Degrees of freedom	Mean square	F ratio
Proteinase	2	1,926.0	16.2[1]
Alpha-amylase	2	9,988.5	84.1[1]
Storage	1	127,653.0	1,075[1]
Proteinase x alpha-amylase	4	25.8	---
Proteinase x storage	2	56.5	---
Alpha-amylase x storage	2	516.5	4.35[1]
Proteinase x alpha-amylase x storage	4	34.5	---
Individual values	196	118.7	
Total	215		

Bread type means

Proteinase	0 -- 112.8, X -- 108.0, 4X -- 102.5
Alpha-amylase	0 -- 121.2, X -- 103.0, 8X -- 99.1
Storage	24 hr. -- 83.5, 94 hr. -- 132.2

$S_{\bar{x}}$ = 8.77[2]

$S_{\bar{x}}$ = 11.57[3]

1 Significant at the 1 percent level.
2 Standard deviation of the difference of two means which will be significant at the 5 percent level.
3 Standard deviation of the difference of two means which will be significant at the 1 percent level.

values obtained for bread to which no proteinase had been added. Compressibility values for bread containing no proteinase and for bread containing 1Y proteinase concentration were not significantly different at the 5 percent level of significance. Likewise, the difference between the values for bread baked with 1Y proteinase concentration and bread baked with 4Y proteinase concentration were not significant. The compressibilities of bread baked with either 1X or 8X concentration of alpha-amylase were significantly (1 percent level) greater than the compressibilities of bread containing no alpha-amylase. At the 5 percent level of significance there was no difference observed between 1X and 8X alpha-amylase compressibilities. The compressibility of the bread after 94 hours of storage was significantly (1 percent level) less than for the bread stored 24 hours.

The results suggest supplementation with an alpha-amylase preparation low in proteinase and a separate proteinase preparation low in alpha-amylase may produce more desirable results for the baker. If such a technic were used, exacting control would be necessary because of the critical nature of proteinase supplementation. When such control measures are not available, an enzyme preparation with a proteinase alpha-amylase activity ratio approximately the same as that for malted wheat flour would probably produce the most desirable results.

Alpha-amylase and Proteinase Activities During Storage

The results from a study on the effect of storage under oxygen, nitrogen and air atmospheres at three different temperatures on the retention of alpha-amylase and proteinase activities of Rhozyme-S supplemented flour are presented in Table 22. Significant differences in the alpha-amylase activities at the different temperatures were observed (Table 23). The curves of Plate II provided a graphical representation of the relationship of alpha-amylase activity retention to time and temperature of storage. Flour stored in the cold room retained the greatest amount of alpha-amylase activity. The alpha-amylase activities fell during the first few months then leveled off. This does not necessarily indicate a serious problem in commercial supplementation.

The gaseous atmospheres did not significantly effect retention of either alpha-amylase or proteinase activity. Both storage temperature and length of storage exerted a marked effect on the retention of proteinase activity. The curves of Plate II indicated storage in the cold room produced less decrease in proteinase activity than storage at either room temperature or at 35°C. With the approach of summer, the temperature in the laboratory increased and for considerable periods was substantially greater than 35°C. This accounts for the intermingling of the room temperature and 35°C. curves.

Table 22. Alpha-amylase and proteinase activity of Rhozyme-S supplemented flour remaining during eight months of storage.

Storage temperature	Atmosphere	Alpha-amylase activity remaining				Proteolytic activity remaining			
		2	4	6	8	2	4	6	8
		%	%	%	%	%	%	%	%
Cold room[1]	O_2	80	80	87	87	92	78	77	68
" "	N_2	94	84	85	81	91	85	88	68
" "	Air	86	87	82	87	91	83	73	68
Room temp.[2]	O_2	81	73	72	79	80	75	62	50
" "	N_2	88	72	75	79	89	75	64	56
" "	Air	83	77	75	81	89	75	66	50
35°C.	O_2	76	72	74	77	85	77	55	59
"	N_2	76	72	70	79	82	77	56	59
"	Air	75	68	73	72	86	75	52	58

1 Approximately 5°C.
2 From 20°C. to 40°C.

Table 23. Analysis of variance of the effect of temperature and gaseous atmosphere upon the retention of alpha-amylase and proteinase activities during storage.

Source of variance	Degrees of freedom	Mean square	F ratio
Alpha-amylase activities			
Atmosphere	2	5	---
Temperature	2	315	23.7[2]
Length of storage	4	857	64.4[2]
Atmosphere x temperature	4	7	---
Atmosphere x length of storage	8	10.1	1.19[1]
Temperature x length of storage	8	29.2	3.44[1]
Atmosphere s lengh of storage x alpha-amylase	16	8.5	
Total	44		
Proteinase activities			
Atmosphere	2	19	1.48[1]
Temperature	2	413	16.6[2]
Length of Storage	4	2,752	11[illegible].5[2]
Atmosphere x temperature	4	9	---
Atmosphere x length of storage	8	6	---
Temperature x length of storage	8	76	5.94[2]
Atmosphere x length of storage x elpha-amylase	16	12.8	
Total	44		

1 Nonsignificant at the 5 percent level.
2 Significant at the 1 percent level.

Microflora Development

The microflora of the supplementation preperations were investigated by determining the number of bacterial spores per

EXPLANATION OF PLATE II

The effect of gaseous atmosphere, temperature of storage, and length of storage on the retention of alpha-amylase activity by a flour supplemented with Rhozyme-3.

Fig. 1. Oxygen atmosphere

Fig. 2. Nitrogen atmosphere

Fig. 3. Air atmosphere

Curve a. 5°C. to 9°C.

Curve b. 20°C. to 40°C.

Curve c. 35°C.

PLATE II

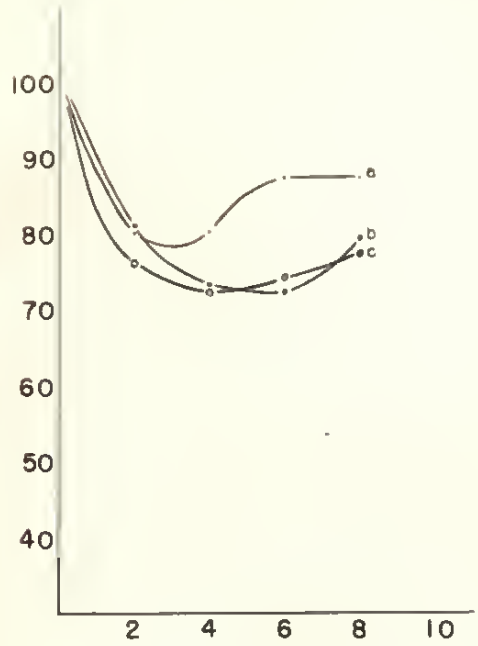


Fig. 1.

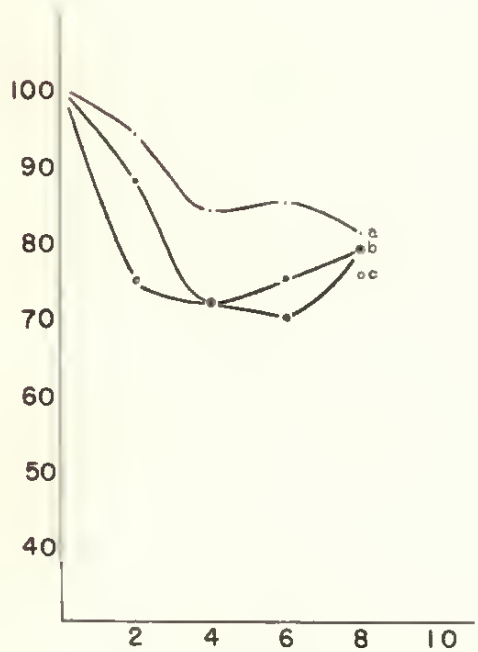


Fig. 2.

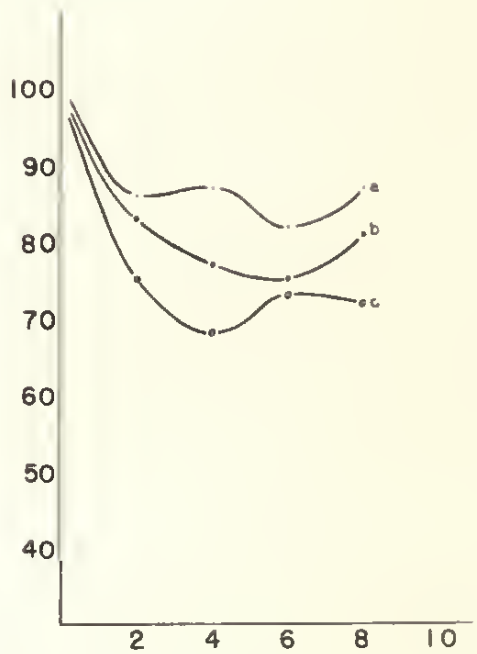


Fig. 3.

PERCENT ACTIVITY REMAINING

MONTHS OF STORAGE

EXPLANATION OF PLATE III

The effect of gaseous atmosphere, temperature of storage, and length of storage on the retention of proteinase activity by a flour supplemented with Thozyme-S.

Fig. 1. Oxygen atmosphere

Fig. 2. Nitrogen atmosphere

Fig. 3. Air atmosphere

Curve a. 5°C. to 9°C.

Curve b. 20°C. to 40°C.

Curve c. 35°C.

PLATE III

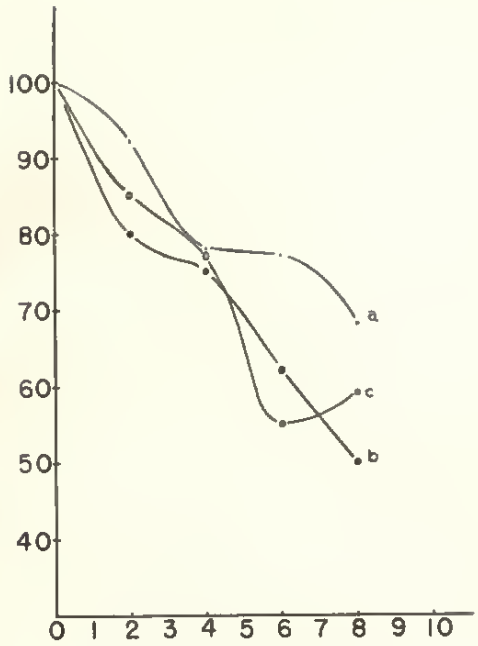


Fig. 4.

PERCENT ACTIVITY REMAINING

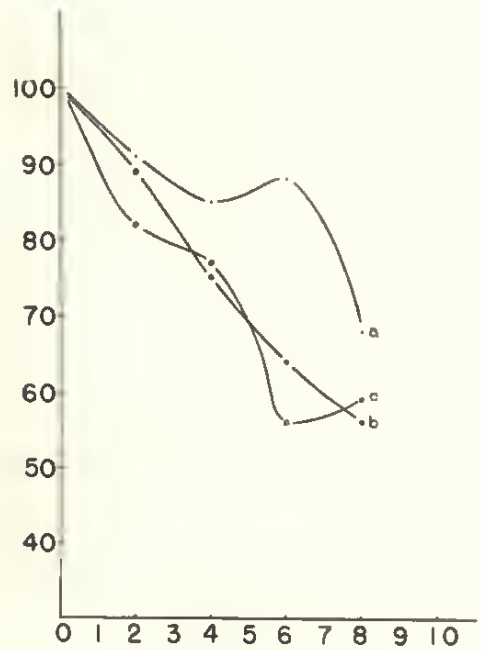


Fig. 5.

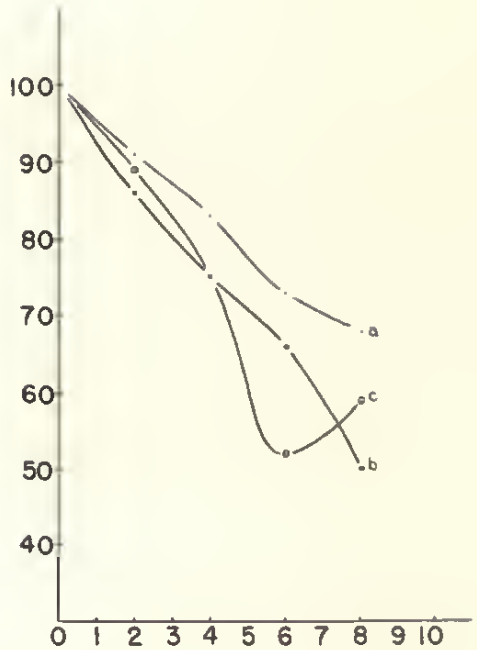


Fig. 6.

MONTHS OF STORAGE

gram of preparation. The results were as follows:

Malted wheat flour	2,100	spores	per	gram
Rhozyme-S	405,000	"	"	"
Diastase-29	1,000,000	"	"	"
Maltase-20	2,500	"	"	"

The counts for Rhozyme-S and Diastase-29 appear very high. However, based on equivalent alpha-amylase activity there is not much difference between Rhozyme-S and the malted wheat flour. In supplementing 100 grams of flour to 1% concentration of alpha-amylase, 535 spores would be added if the malted wheat flour was used, while 555 spores would be added if Rhozyme-S was added. In supplementing 100 grams of flour to 1% concentration with Diastase-29, 2,350 spores would be added.

From the criticism of James and Smith (10) of the present A.A.C.C. method of determining rope spore counts, it was concluded that these results merely gave presumptive indication of the number of rope spores present. However, rope development in bread baked with either Rhozyme-S, Maltase-20 or Diastase-29 and stored up to eight days under favorable conditions was not observed.

While the results of this investigation indicated fungal enzyme preparations may be used as commercial alpha-amylase supplements for flour, the approval of the Pure Food and Drug Administration must be secured before such preparations may be utilized for commercial flour supplementation.

SUMMARY AND CONCLUSIONS

1. Comparison of the alpha-amylolytic and proteolytic activities of eight enzyme concentrates indicated that an enzyme concentrate could be obtained which would possess any desired proteinase to alpha-amylase ratio.

2. Variance of dextrinogenic activities of a series of Rhozyme-S samples suggests enzyme concentrates should be standardized on dextrinogenic activity.

3. Rhozyme-S, Maltase-20, Diastase-33 and Diastase-34 supplementation may be used to produce bread comparable in quality to that produced with malted wheat flour. Diastase-29 and Diastase-32 liquified the sponges if used in concentrations sufficient to produce beneficial results from the alpha-amylase. The bacterial preparations, Diastase-28 and -30, were undesirable as alpha-amylase supplements for flour since they caused sticky and gummy bread crumb.

4. The alpha-amylases of various bacterial enzyme concentrates may vary in thermostability.

5. Increasing the temperature from 30°C to 50°C caused the alpha-amylase of malted wheat flour to increase more in activity than either Diastase-28 or Diastase-30.

6. Equivalent amounts of Diastase-28 and -30 (based on dextrinogenic activity at 30°C) produced maximum viscosity values significantly lower than the values for malted wheat flour. Thus, equivalent amounts of Diastase-28 and -30 (bacterial) alpha-amylase apparently cause greater starch degradation than an equiva-

lent amount of malted wheat flour alpha-amylase even though thermostability and increase in activity at higher temperatures date indicate malted wheat flour would produce the greatest starch degradation. This apparent anomaly may be explained by assuming a lesser affinity of bacterial alpha-amylase for lower molecular weight dextrins.

7. Pilot plant baking studies with Rhozyme-S and Maltase-20 confirmed the experimental baking suggestion that these two preparations may be used on a commercial scale to produce bread comparable in quality to the bread produced with malted wheat flour.

8. Pilot plant bakery investigation demonstrated that it would be feasible to dilute commercial enzyme preparations with flour to the alpha-amylase activity of commercial malted wheat flour, thus, the malt feeders now in use in flour mill could be used to blend in the diluted amylase preparations.

9. The desirability of reducing the proteinase activity of Rhozyme-S was demonstrated by baking with Rhozyme-S extracts in which various amounts of proteinase had been inactivated.

10. Investigation of the separate effect of alpha-amylase and proteinase supplementation corroborated the work of Read and Haas (21), that various flours may be improved by increments of proteinase enzyme concentrate.

11. The level of protsinase supplementation was found to be quite critical while the amount of alpha-amylase supplementation could be varied considerably without causing excessive detrimental effects.

12. Increasing amounts of proteinase and or alpha-amylase, increased the bread crumb compressibility.

13. Gaseous atmospheres did not significantly affect the retention of alpha-amylase and proteinase activities. The higher temperatures significantly decreased both alpha-amylase and proteinase activities below that of the activities retained by storage in the cold room. The proteinase activities consistantly decreased during storage. While significant decrease in alpha-amylase activity was found, a serious problem in commercial supplementation is not necessarily indicated.

14. Commercial enzyme preparations would not necessarily introduce more bacterial spore contamination than would malted wheat flour supplementation when compared on equivalent alpha-amylase concentration.

15. The approval of the Pure Food and Drug Administration must be secured before fungal enzyme concentrates may be used for commercial flour supplementation.

ACKNOWLEDGMENTS

Acknowledgment and sincere thanks are extended to Mr. John A. Johnson, major instructor and Associate Professor of Milling Industry, and to Dr. Byron S. Miller, Associate Professor of Milling Industry, for invaluable aid in directing this investigation and for counsel and criticism in the preparation of this manuscript. Thanks are due Mr. W. S. Taylor, Department of Bacteriology, for making the bacterial spore count determinations.

Appreciation is expressed to Dr. John A. Shellenberger, Head, Department of Milling Industry, for providing research facilities; to Dr. Max Milner for counsel, and to other members of the Department of Milling Industry without whose co-operation this investigation could not have been completed.

The author is indebted to Rohm and Haas Company, Philadelphia, Pennsylvania, for the financial assistance which has made this investigation possible and for furnishing the commercial enzyme preparations.

Thanks are due the following companies for contributing various types of flour for the investigation: Bay State Milling Co., Winona, Minnesota; The Larabee Flour Mills Company, Kansas City, Missouri; Montana Flour Mills Company, Great Falls, Montana; and Rodney Milling Company, Kansas City, Missouri. Thanks are also due Kansas Milling Co., Wichita, Kansas, for furnishing the malted wheat flour.

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