A METHOD FOR THE DETERMINATION OF RELATIVE AMOUNTS OF MALTED-WHEAT, FUNGAL (Aspergillus oryzae) AND BACTERIAL (Bacillus subtilis) ALPHA-AMYLASE IN MIXTURES, AND ITS APPLICATION TO MALTED WHEAT[1]

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ABSTRACT

A method to determine relative amounts of malted-wheat, fungal (*Aspergillus oryzae*), and bacterial (*Bacillus subtilis*) alpha-amylase activities in mixtures is presented. The method depends on differences in the thermostability of the three types of amylases and the use of simultaneous algebraic equations to calculate the percentages. Results of analyses of known mixtures of the three sources agree well with known composition.

The presence of fungal alpha-amylase in malted wheat was closely related to the observed development of molds. There was little bacterial alpha-amylase in any of the malted wheats. The evidence suggests that contribution of the fungus to the total alpha-amylase activity of malted wheat is due to the production of fungal alpha-amylase, and not to gibberellic acid which could stimulate the production of cereal alpha-amylase. Up to 15% of the amylolytic activity of moldy malted wheat was due to fungal alpha-amylase.

Infestation of cereals by microorganisms is almost universal, and the moist conditions prevalent during malting are optimal for development of the hydrophilic segment of microflora. Most prior studies have been concerned mainly with detecting (13) and controlling (4,9) microorganisms during malting and the effects of selected organisms on malt properties (11). Little information is available concerning the amounts of alpha-amylase contributed by fungi and bacteria. Knight (7) used the difference in the thermostability of fungal and cereal alpha-amylase to detect and estimate in other than SKB units the amount of fungal alpha-amylase which had been added to flours.

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No method which permits the detection of bacterial alpha-amylase in mixtures has been found in the literature.

Miller, Johnson, and Palmer (8) compared the thermostability of fungal, bacterial, and malted wheat flour alpha-amylase. Bacterial *(Bacillus subtilis)* alpha-amylase was the most thermostable, cereal alpha-amylase possessed intermediate thermostability, and fungal *(Aspergillus oryzae)* alpha-amylase was the least thermostable. The purpose of the present study has been to devise a procedure for the determination of relative amounts of cereal, fungal, and bacterial alpha-amylase in mixtures and to apply this method to malted wheat.

Materials and Methods

The sources of alpha-amylase used in this investigation consisted of several experimentally produced wheat malts (3), a fungal preparation (*Aspergillus oryzae*),[2,3] and a bacterial preparation (*Bacillus subtilis*)[2]. A standard malted wheat essentially free of mold was prepared by steeping the grain in 0.05% formaldehyde solution during the last hour of the steep period before germination (4).

Malts of Redlan sorghum, Kanota oats, Kindred barley, an unnamed white corn, and a hard red winter wheat were prepared under comparable conditions (germinated at 62°F. for 4 days) in order that the thermostability of alpha-amylases of these cereals could be compared. The alpha-amylase activities of these five malted cereals were 90, 68, 149, 51, and 161 SKB units per g., respectively.

Alpha-amylase activity was determined by a modified Wohlgemuth procedure (method IA) of Hagberg (5). This procedure makes possible the rapid detection of small quantities of alpha-amylase and the expression of activities in terms of the familiar SKB units.

The procedure was as follows: Extracts of malted-wheat, fungal and bacterial alpha-amylases were diluted to 470 ml. with distilled water and 25 ml. of a sodium acetate-acetic acid buffer. The buffer was made by mixing 40 ml. of a 4.0 molar solution of glacial acetic acid with 460 ml. of a 4.0 molar solution of anhydrous sodium acetate. The final pH of the buffered enzyme solutions, which also contained 0.2% calcium chloride, was 5.55. The initial alpha-amylase activity of the mixtures ranged from 50 to 600 SKB units per 470 ml. of liquid. Fifty grams of flour were added to a duplicate series of the diluted extracts because of the known stabilizing effect of substrates

[2] Rhozyme-33 is a fungal and Rhozyme-39 a bacterial concentrate. (Rohm and Haas Co., Philadelphia, Pennsylvania.)

[3] The mention in this publication of a trade product, equipment, or a commercial company does not imply its endorsement by the U.S. Department of Agriculture over similar products or companies not named.

on certain enzymes (8). Both series of extracts were heated in an amylograph and aliquots were removed at 30° and at 5°C. intervals from 60° to 95°C.

Aliquots were immediately chilled in an ice-water bath, centrifuged when necessary, and analyzed for alpha-amylase.

Results and Discussion

The data in Table I, representing the average of eight replicate

TABLE I

RELATIVE THERMOSTABILITY OF MOLD-FREE MALTED-WHEAT, FUNGAL, AND BACTERIAL ALPHA-AMYLASES

TEMPERATURE	ENZYME RETAINED IN PRESENCE OF FLOUR			ENZYME RETAINED WITH NO FLOUR PRESENT		
	Malted-Wheat	Fungal	Bacterial	Malted-Wheat	Fungal	Bacterial
°C	%	%	%	%	%	%
65	100	100	100	100	100	100
70	100	54.0	100	100	53.6	100
75	58.0	5.0	100	60.0	3.7	100
80	28.0	1.6	100	26.0	1.0	94
85	2.1	..	95.0	1.6	..	60
90	..	..	74.0	..	..	21
95	..	..	18.0	..	..	7
Std. Dev.						
65	0.8	0.4	0.8	1.0	0.4	0
70	0.2	2.5	0.8	1.8	2.6	2.5
75	3.1	0.2	1.0	2.6	0.5	2.0
80	3.4	0.1	0.4	2.8	1.3	1.7
85	0.4	..	3.5	0.3	..	3.6

experiments, indicate a pronounced difference in the stability of the various alpha-amylases to heat. Fungal alpha-amylase was the most thermolabile and the bacterial enzyme the most stable. The data agree with those reported previously (8) when this same procedure was employed. As also shown previously, the presence of flour had relatively little effect on the thermostability of wheat-malt and fungal amylases, but had a pronounced protective effect on the bacterial amylase at temperatures above 80°C. Adding flour to all enzyme extracts was adopted as a routine practice in this study.

Knight (7) reported a measurable decrease in wheat-malt alpha-amylase heated at 68°C. for 30 minutes. In the present work, there was no decrease at 70°C. This difference is probably due to the rapid heating (1.5°C. per minute) in the amylograph. It could be partly due to the presence of fungal amylase in the malt used by Knight (7). In the present work, fungi were eliminated by the use of a formaldehyde treatment (4).

The data in Table I suggest that it should be possible to determine the relative amounts of each enzyme by the use of simultaneous equations. The percentages of each type of alpha-amylase remaining after heating at three temperatures are used as coefficients. The activity of all three alpha-amylases was 100% at 65°C., thus:

$$C + F + B = \text{total activity}$$

where C represents the contribution of cereal (wheat-malt) alpha-amylase, F that of fungal alpha-amylase, and B that of bacterial alpha-amylase to the total activity. At 70°C. only 54% of the fungal alpha-amylase is retained, thus:

$$C + 0.54\,F + B = \text{total activity}$$

Similarly, at 75°C., 58% of the cereal and 5% of the fungal alpha-amylase are retained, thus:

$$0.58\,C + 0.05\,F + B = \text{total activity}$$

The amount of each type of alpha-amylase present in a given enzyme mixture may be determined by solving for the unknowns in the three simultaneous equations. Since the thermostability of malted-wheat alpha-amylase may be somewhat different from that of other cereals, thermostability data for the amylase in these cereals should be obtained before attempting to apply this method.

The proposed method was tested by analyzing mixtures of the three enzymes. The amount of each component varied from 5 to 90% of the total. The data given in Table II, which were obtained by

TABLE II

MEASURED AND CALCULATED AMOUNTS[a] OF MALTED-WHEAT, FUNGAL, AND BACTERIAL ALPHA-AMYLASE IN KNOWN MIXTURES

Total Activity	Mold-Free Malted-Wheat		Fungal		Bacterial	
	Added	Found	Added	Found	Added	Found
SKB units	%	%	%	%	%	%
156	80.0	81.8	15.0	15.4	5.0	2.8
146	80.0	78.8	10.0	10.4	10.0	10.8
155	75.0	65.8	5.0	4.2	25.0	30.0
153	60.0	56.5	20.0	21.3	20.0	22.2
150	50.0	49.0	30.0	30.6	20.0	20.4
156	50.0	47.7	20.0	20.9	30.0	37.4
150	33.3	31.5	33.3	33.3	33.3	35.2
156	30.0	28.5	30.0	30.8	40.0	40.7
156	20.0	23.7	40.0	40.4	40.0	35.9
150	10.0	12.0	45.0	43.5	45.0	44.5
146	5.0	9.5	45.0	41.8	50.0	48.7
154	10.0	9.2	80.0	80.5	10.0	10.3
146	20.0	20.8	60.0	58.2	20.0	21.0
150	10.0	10.0	10.0	10.1	80.0	79.9
153	20.0	20.9	20.0	19.6	60.0	59.5

[a] Percent of total alpha-amylase activity.

TABLE III

RELATIVE AMOUNTS OF MALTED-WHEAT, FUNGAL, AND BACTERIAL ALPHA-AMYLASE IN MALTS DIFFERING IN THEIR CONTAMINATION WITH MOLD

MOLDINESS OF MALT SAMPLE	TOTAL ALPHA-AMYLASE ACTIVITY			SOURCES OF ALPHA-AMYLASE ACTIVITY		
	At 65°C.	At 70°C.	At 75°C.	Malted-Wheat	Fungal	Bacterial
	SKB units/g	*SKB units/g*	*SKB units/g*	%[a]	%[a]	%[a]
Slight	140.0	138.4	79.2	97.2	2.5	0.3
Slight	148.0	145.5	83.0	96.0	3.7	0.3
Slight	145.0	140.3	78.7	92.2	7.1	0
Moderate	145.0	140.8	79.7	93.1	6.3	0.6
Heavy	139.7	133.0	73.3	89.5	10.4	0.1
Heavy	152.7	145.0	79.8	88.7	10.9	0.4
Very heavy	163.3	152.2	82.2	84.9	14.8	0.3

[a] Percent of total activity.

more than one operator at different times, show the distribution of the three sources of alpha-amylase in the mixtures. The amount of alpha-amylase "found" in each mixture in most instances agreed with the "known" amount within acceptable experimental limits. Since the activity of fungal amylase decreases very rapidly between 65° and 75°C. and that of malt amylase between 75° and 85°C., samples must be withdrawn and cooled rapidly according to the same fixed experimental procedure for both known and unknown sample mixtures.

The data in Table III show the relative amounts of cereal, fungal, and bacterial alpha-amylase in a series of malted wheats which differed in apparent moldiness. The most heavily infested samples were produced deliberately by introducing a small quantity of moldy wheat at the start of the germination period. These malts were comparable in moldiness to some other samples produced in a prior investigation (3). In general, the malts which appeared to be the least contaminated by mold contained the least fungal amylase. The amount of bacterial alpha-amylase found was slight in all instances. This finding agrees with observations made during an earlier study (4) when malts were tested for microorganisms by use of sterile malt agar plates.

The relative amounts of fungal alpha-amylase found in malted wheats (Table III), particularly those in which mold growth was moderate to heavy, agree with data by Prentice and Sloey (11) which were obtained after intentionally adding fungi during malting. While Sheneman and Hollenbeck (13) found appreciable numbers of bacteria present during malting of barley, Prentice and Sloey (11) found that bacteria added deliberately during the malting process caused no significant increase in alpha-amylase activity of finished malt.

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