ALPHA-KETO ACIDS IN BREAD PRE-FERMENTS[1]

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ABSTRACT

Eight different alpha-keto acids were detected in cell-free pre-fermented broths similar to those used in commercial bread-baking. Three of the acids, alpha-ketoglutaric, pyruvic, and alpha-ketoisovaleric, occurred in amounts large enough to measure quantitatively. The concentrations of these three acids in the pre-ferment increased with time of incubation. At the end of 6 hr., the accumulation of pyruvic acid was largest. The remaining five keto acids occurred in such small amounts that paper chromatography could not easily resolve their 2,4-dinitrophenylhydrazones.

Liquid pre-ferments have become important in industrial baking because they adapted easily to continuous dough mixing (1). One serious disadvantage of such fermented mixtures, however, is that they do not easily give intense flavor or aroma; consequently, the composition of pre-ferments has been subjected to study in an attempt to identify those flavor precursors that give rise to the characteristic bread aroma during baking.

Among possible flavor precursors in pre-ferments are the carbonyl compounds, some of which have been isolated and identified by various workers (2,3,4). To date most of this work has been confined to the identification of ketones and aldehydes. The alpha-keto acids have received little attention. Analyses of pre-ferments and yeast cultures similar to pre-ferments have shown that pyruvic, alpha-ketoglutaric, and alpha-ketobutyric acids are present in appreciable quantities at the end of fermentation (5,6).

The presence of these keto acids prompted a search for similar compounds. The present study revealed several other keto acids, many in small quantities. They have been identified, and the acids in highest concentrations in the pre-ferment have been measured quantitatively.

Materials and Methods

Reagents. Bakers' yeast was obtained commercially. Carbonyl-free methanol was prepared by treating reagent grade methanol with 2,4-dinitrophenylhydrazine (DNP) in acid solution and then distilling. Ethyl acetate was treated with 0.1*N* sodium bicarbonate and then

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washed with distilled water until washings were neutral to litmus. Petroleum ether was shaken with concentrated sulfuric acid and allowed to stand about 2 weeks. After standing, the solvent was washed with 1*N* sodium carbonate and then with distilled water, and finally distilled.

DNP was crystallized from carbonyl-free methanol. DNP reagent was prepared fresh immediately before use by dissolving 0.1 g. DNP in 100 ml. of 2*N* hydrochloric acid.

Alpha-ketoglutaric and pyruvic acids were obtained commercially. Alpha-ketoisovaleric acid was synthesized by oxidative deamination of valine according to the procedure of Meister (7).

2,4-Dinitrophenylhydrazone (DNPH) derivatives of the keto acids were prepared by dissolving the appropriate keto acid in water and adding excess DNP reagent. The precipitated derivative was recrystallized from hot water.

Preparation of Pre-Ferment. The composition of the pre-ferment is described in a previous paper (5). It contained 11.9% sucrose and 7.0% bakers' yeast. Brew fermentation lasted from 1 to 6 hr. at 35°C. At the end of each hour, 50 ml. was withdrawn and centrifuged in a refrigerated centrifuge to remove yeast cells. The supernatant was quickly frozen and stored until ready for use.

Identification of the Keto Acid DNPH. One liter of pre-ferment supernatant reacted with 700 ml. of the DNP reagent at room temperature for 30 min. The keto acid DNPH was then extracted from the mixture, according to method II of Kawano *et al.* (8).

The keto acids were identified by catalytically reducing their DNPH derivatives to amino acids by the procedure of Linko and Milner (9). The amino acids were then separated on a column of Dowex 50 resin and analyzed qualitatively on a Beckman-Spinco Model 120 Analyzer (10). Additional analyses for amino acids were made by paper chromatography (11). The keto acid-DNPH mixture was also subjected to paper chromatography (12). Each hydrazone spot that separated was cut out, eluted with 0.1*N* sodium bicarbonate, and hydrogenated. The resulting amino acid was identified by paper chromatography as before.

For the quantitative analysis of the keto acids, 0.5 to 1 ml. of pre-ferment was treated with 100 ml. of DNP reagent, and the derivatives were extracted from the pre-ferment essentially by the method I of Kawano *et al.* (8). In addition, the ethyl acetate layer was passed through a column of Dowex 50 (hydrogen form) according to the method of Schwartz (13) to remove the excess DNP from the derivative.

Ethyl acetate (3 to 4 column volumes) was then passed through to remove the derivatives completely. At the end of this extraction proce-

dure the sodium bicarbonate layer which contained the derivative was made acidic with 2*N* hydrochloric acid and washed with 15 ml. ethyl acetate. The ethyl acetate containing the derivatives was evaporated to a volume of about 2 ml.; from 5 to 300 μl. were spotted on paper and run according to the previously cited procedure (12). After separation, the spot containing the keto acid DNPH was cut out, broken up in a small beaker with 3 ml. of 1*N* sodium hydroxide, and filtered through a sintered glass funnel. The paper on the funnel was rinsed with enough sodium hydroxide to bring the filtrate to 6 ml. The absorbance of this solution was then measured at 510 mμ. With this solvent system pyruvic acid DNPH always resolved into two spots (*syn* and *anti* forms) which were combined and measured during each determination. Standard calibration curves were prepared by dissolving the appropriate keto acid DNPH in ethyl acetate and adding known amounts of this solution to colorimeter tubes. The ethyl acetate was evaporated from these tubes in vacuum, and 6 ml. of 1*N* sodium hydroxide was added to the residue. The absorbance was then measured. To test the efficiency of these extraction and chromatographic steps, determinations were made on the DNPH's of the appropriate authentic keto acids in place of the pre-ferment sample containing the keto acids.

Results and Discussion

Table I shows the amino acids that resulted from the hydrogenation of the mixture of keto acid 2,4-DNPH derivatives prepared from the pre-ferment. Three amino acids — alanine, glutamic acid, and valine — occurred in sizable amounts. These originated from the derivatives of pyruvic, alpha-ketoglutaric, and alpha-ketoisovaleric acid, respectively. The concentration of these amino acids, however, can be considered only an approximation of the concentration of the keta acids present, since previous workers (9,14) have shown that the hydrazone derivative does not convert quantitatively to its amino acid analog upon hydrogenation.

TABLE I

KETO ACIDS FOUND IN THE PRE-FERMENT

KETO ACIDS	AMINO ACIDS OBTAINED BY HYDROGENOLYSIS	KETO ACIDS	AMINO ACIDS OBTAINED BY HYDROGENOLYSIS
Pyruvic	alanine	*p*-Hydroxyphenyl-pyruvic	tyrosine
Alpha-ketoglutaric	glutamic acid	Alpha-ketoisocaproic	leucine
Alpha-ketoisovaleric	valine	Alpha-keto-beta-methylvaleric	isoleucine
Beta-hydroxypyruvic	serine		
Alpha-keto-gamma-methiolbutyric	methionine		

The remaining five keto acids occurred in the pre-ferment in very low concentrations, and paper chromatography did not easily resolve their DNPH derivatives. The hydrazones of these acids appeared to move on paper at a rate close to that of alpha-ketoisovaleric DNPH. Meister and Abendschein (15) reported R_F values for most of these derivatives similar to that of alpha-ketoisovaleric acid. In order to detect the derivatives of the minor acids, the chromatograms had to be overloaded with the hydrazone mixture. When this was done, however, the large quantities of pyruvic and ketoisovaleric derivatives covered the minor keto acids completely.

In this work, when the spot containing the alpha-ketoisovaleric DNPH was eluted from paper and hydogenated, the product consisted mostly of valine along with traces of other amino acids. Thus the values for alpha-ketoisovaleric acid reported here should be considered semiquantitative measures of its concentration in the pre-ferment. However, most of the hydrazone color originated from the DNPH of alpha-ketoisovaleric acid.

The efficiency of the extraction and separation of the keto acid DNPH derivatives was determined by recovery experiments at the same levels of keto acid concentration as those in 6-hr. pre-ferments. The recovery of the derivatives of the three keto acids that were determined quantitatively (see Table II) was: pyruvic, 99%; alpha-ketoglutaric, 93%; and alpha-ketoisovaleric, 90%.

Table III shows the production of the three main keto acids with time of fermentation. Pyruvic acid was produced in much higher con-

TABLE II

RECOVERY OF DNPH'S OF KETO ACIDS

KETO ACID	R_F VALUE OF DNPH[a]		RECOVERY OF DNPH	
	Authentic	Pre-Ferment	Amount Added[b]	Amount Recovered
Pyruvic	52;67	52;67	1.75	1.73
Alpha-ketoglutaric	15	16	0.364	0.338
Alpha-ketoisovaleric	79	79	0.543	0.495

[a] R_F multiplied by 100 determined by the method of McArdle (12).
[b] Micromoles of DNPH derivative.

TABLE III

PRODUCTION OF MAJOR KETO ACIDS IN PRE-FERMENTS[a]

KETO ACID	HOURS						
	0	1	2	3	4	5	6
Alpha-ketoglutaric	0	0.17	0.20	0.22	0.25	0.26	0.25
Pyruvic	0.11	7.1	14.0	18.0	20.0	19.0	17.0
Alpha-ketoisovaleric	0	0.21	0.48	0.80	0.74	0.62	0.78

[a] Millimoles per liter of pre-ferment.

centrations than alpha-ketoglutaric and alpha-ketoisovaleric acids. This high level of pyruvic acid in the fermenting medium has been reported by other workers to be due to a deficiency of decarboxylase in the yeast cell (16). The keto acids that occur in small quantities in the pre-ferment could contribute to bread flavor and aroma, particularly the sulfur-containing acid, alpha-keto-gamma-methiolbutyric acid. Evans *et al.* (17) have commented upon the large number of cases in which nitrogen and sulfur compounds, particularly the latter, appear to give flavor.

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