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Studies on Malt Amylase. (IV)

The Effect of Organic Acids with Gibberellin on Alpha-Amylase Development in Wheat Seeds

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Several investigators have demonstrated that germination and alpha-amylase synthesis in seed are stimulated by treatment with gibberellin (GA), but are inhibited by organic acids. The effect of steeping wheat seed in GA solutions containing organic acids at various concentrations and pH levels on alpha-amylase development in seed was investigated. The inhibitory effect of fatty acids on the ability of GA to develop alpha-amylase increased with lengthening the chain of acid. Acetic acid, sodium acetate and acetate buffer (pH 4.7) solutions showed stimulatory effects at lower, but inhibitory effects at higher concentrations. The effect of acetic acid was similar on both of germinated and non-germinated seeds. Fumaric, malonic and malic acids were the least inhibitory when acids were used at higher concentrations. It appeared that the effect of organic acid on GA to develop alpha-amylase in seed depended upon the kind of acid rather than pH.

INTRODUCTION

Since Hayashi[1] reported that gibberellin (GA) increased the amount of enzyme activity in germinated cereal grains, numerous workers have studied the response of cereal grains to treatment with GA during the malting process. Macey and Stowell[2] reported that combination of GA and potassium bromate was effective in increasing enzyme activities, and in curtailing malt loss. Similar results were obtained when 2, 4-dichlorophenoxyacetic acid, 2(3)-benzoxazolone and Roccal (alkyldimethylbenzylammonium chloride) were used in combination with GA.[3,4,5] The author[6] demonstrated that simultaneous or subsequent use of hydrogen peroxide with GA enhanced the effect of GA. Mastovsky[7] reported that combinations of glucose and GA were more effective than GA alone. A number of organic acids have been detected in cereal grains when they are thought to play significant roles in germination and metabolism.[8,9] Cook and Pollock[10,11] also found that some carboxylic acids are inhibitory on germination of seed grain. Comparatively few attempts have been made, however, to use organic acids with or without GA during malting. Menzel[12] reported that high enzyme activity malts were produced when the grain was washed with dilute acids. Fleming, Johnson and Miller[13] demonstrated that low concentrations of fumaric and succinic acids with GA stimulated enzyme production in malting. The present work investigates the effect of several organic acids on the stimulatory effect of GA to produce alpha-

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amylase in wheat.

EXPERIMENTAL

Triumph (1962 crop) and Omar (1961 crop) wheat seeds were used for the experiment. Ten grams of seed were presteeped in 25 ml of 0.2% sodium hypochlorite for 5 minutes for the purpose of surface sterilization, then washed with water and steeped in 50 ml of water for 1 hour. The seeds were resteeped in 25 ml of 0.001% GA solution containing various concentrations of organic acids for 20 hours at room temperature. The pH values of steeping solution were determined at the beginning and at the end of the steeping period with a Beckman pH meter. After being steeped, the solutions were drained away and the seeds incubated for 3 days at 17°C on water moistened filter paper in petri dishes. The malts were kilned in an air-convection oven at 40°C for 24 hours. The kilned seeds were ground and extracted with 0.2% $CaCl_2$ solution for the determination of alpha-amylase activity (SKB-units) by Sandstedt, Kneen and Blish procedure.[14] SKB units per gram were calculated on dry basis. Contaminated seeds were detected microscopically and discarded.

Potassium gibberellate (GA) employed were a product of Merck & Co., Rahway, New Jersey, U.S.A.. Organic acids used were commercially prepared.

RESULTS AND DISCUSSION

Cook and Pollock[11] demonstrated that water in which barley had been steeped for as little as 4 hours contained a germination inhibitor and that an appreciable amount of acetic acid was present after 24 hours of steeping. Laties[15] stated that acetate had an immediate deleterious influence on the respiratory cycle of

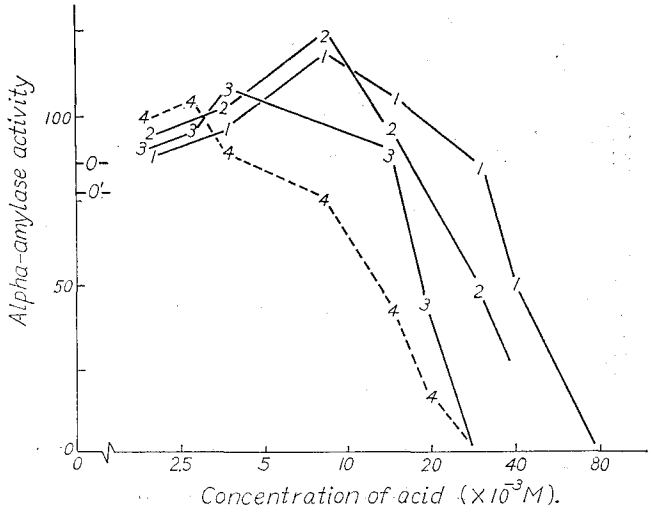

Fig. 1. Effect of steeping in GA and acetate on alpha-amylase development in wheat. Control: Steeped in GA only. 0; Control, 0'; Control (non-germinated), 1; Acetate buffer (pH 4.7), 2; Sodium acetate, 3; Acetic acid, 4; Acetic acid (non-germinated).

barley. In preliminary experiments, the author confirmed that steeping wheat seeds in acetic acid, sodium acetate and acetate buffer (pH 4.7), at low concentrations, inhibited germination but slightly stimulated alpha-amylase development in the seed. It, thus, seemed likely that acetate ion had a characteristic influence on germination and enzyme production of seed.

The effects on alpha-amylase development of steeping Triumph wheat in GA solution containing acetic acid, sodium acetate and acetate buffer (pH 4.7) are shown in Fig. 1. Steeping in concentrations in excess of 0.016 M of acetic acid or sodium acetate or 0.032 M acetate buffer inhibited alpha-amylase development. Lower concentrations, however, showed stimulatory effects. It is likely that these results were not due to hydrogen-ion concentration of the steeping solutions used, since the initial pH values of the steeping solutions were 3.1–3.8 with acetic acid, 6.5–7.2 with sodium acetate and 4.7 with acetate buffer. To determine whether the observed changes in alpha-amylase production were directly linked with germination, the seeds steeped in GA-acetic acid were frozen at −12°C for 24 hours to prevent germination. The treated seeds were then incubated for 3 days as were other germinated seeds. Data for the non-germinated seeds (Fig. 1) indicate that acetic acid was inhibitory at higher, but stimulatory at lower concentrations as in the case with germinated seeds. Paleg[16] demonstrated that 0.01 M acetate buffer of pH 5.5 completely suppressed the sugar release promoted by GA in excised barley endosperm.

The effects of steeping in GA containing various concentrations of fatty acids on alpha-amylase development in Omar wheat seeds are shown in Fig. 2. Low concentrations of formic and acetic acids had a stimulatory effect on the GA function to develop alpha-amylase. The inhibitory effect of the acid used at the same concentrations increased with length of the fatty acid chain. *n*-Pelargonic acid had the most inhibitory effect at the low concentration (0.002 M) but was

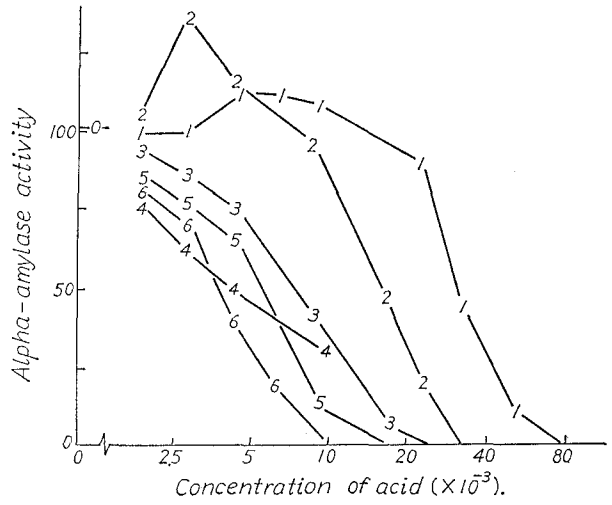

Fig. 2. Effect of steeping in GA and acid on alpha-amylase development. 0; Control, 1; Formic acid, 2; Acetic acid, 3; Propionic acid, 4; Pelargonic acid, 5; Butyric acid, 6; Caproic acid.

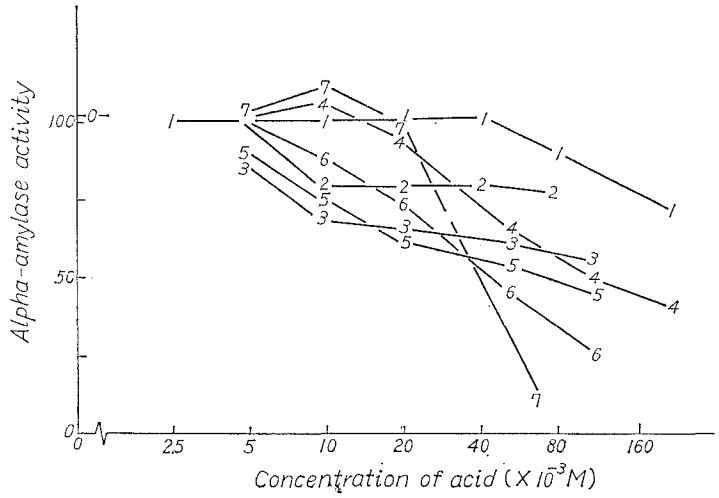

Fig. 3. Effect of steeping in GA and acids on alpha-amylase development (Omar wheat).
0; Control, 1; Malic acid, 2; Fumaric acid, 3; Citric acid, 4; Malonic acid, 5; Succinic acid, 6; Pyruvic acid, 7; Lactic acid.

not so inhibitory as butyric and caproic acids at higher concentrations, probably because of the low solubility of *n*-pelargonic acid. Mosolov and Afanasev[17] demonstrated that the inhibitory effect of fatty acid on trypsin activity increased with the chain length of the acid. The effect may have been due to the denaturation of the enzyme, but the effect of acid in the present experiment may be due to more complicated processes. In Fig. 3, at low concentrations, citric acid was the most inhibitory and was followed by succinic, fumaric, pyruvic, malic, malonic and lactic acids, in that order. At higher concentrations, however, lactic

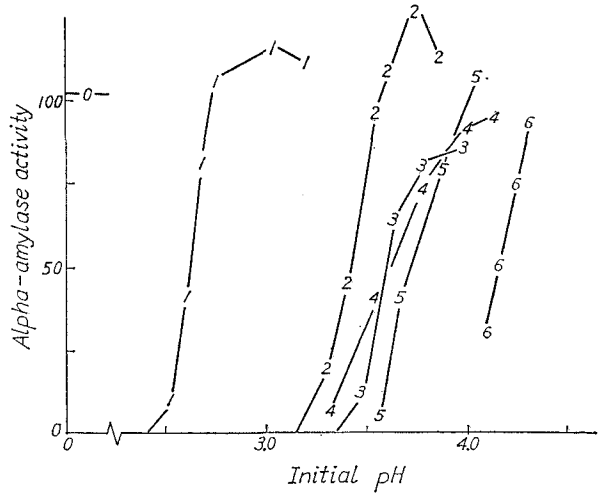

Fig. 4. Relationship between initial pH of steeping solution and alpha-amylase development (Omar wheat).
0; Control, 1; Formic acid, 2; Acetic acid, 3; Butyric acid, 4; Propionic acid, 5; Caproic acid, 6; Pelargonic acid.

Table 1. The changes of pH of steep solution during steeping.

Acid or salt added	Concentration	pH of steeping solution Initial	Final
	(M)		
Triumph Wheat			
Control (Water)	—	5.8	5.6
Acetic	0.004	3.7	5.2
	0.030	3.2	4.3
Na-acetate	0.008	6.9	5.7
	0.030	7.2	5.9
Acetate-buffer	0.008	4.7	5.4
	0.080	4.7	5.0
Omar Wheat			
Control (Water)	—	5.8	5.5
Formic	0.005	3.1	4.5
	0.080	2.4	3.2
Acetic	0.003	3.8	5.3
	0.030	3.2	4.3
Propionic	0.002	4.0	5.2
	0.016	3.3	4.3
n-Butyric	0.001	4.0	5.4
	0.020	3.3	4.3
n-Caproic	0.001	4.1	5.5
	0.010	3.6	4.7
n-Pelargonic	0.001	4.3	5.6
	0.010	4.1	5.3
Pyruvic	0.004	3.1	4.7
	0.050	2.3	2.8
Lactic	0.010	2.8	3.7
	0.100	2.4	2.9
Citric	0.005	2.9	3.5
	0.100	2.2	2.4
Succinic	0.005	3.8	4.5
	0.100	2.8	3.3
Fumaric	0.005	2.9	4.0
	0.060	2.3	2.6
Malic	0.005	3.0	4.5
	0.200	2.0	2.3
Malonic	0.010	2.6	3.2
	0.200	1.9	2.1

acid was the most inhibitory, followed by pyruvic, succinic, malonic, citric, fumaric and malic acids.

The relationship between initial pH of steeping solutions containing GA and the fatty acids, and alpha-amylase development is shown in Fig. 4. Table 1 shows the changes in pH of the steeping solution while wheat was steeped in GA and the acids. Concentrations in the table represent those that caused development of the highest and the lowest alpha-amylase activities in the range of acids examined. Inhibitory effects of the fatty acids at a low pH increased with the

chain length of acid (Fig. 4). Formic acid caused increased alpha-amylase production in seed, as the initial pH of 2.4-3.1 changed to 3.2-4.5 at the end of steeping. *n*-Pelargonic acid induced the most inhibitory effect on the development of enzyme, though its initial pH of 4.1-4.3 changed to 5.3-5.6 during steeping time. Data of Fig. 3 and Table 1 indicate that succinic acid was the most inhibitory in the vicinity of pH 3.0, and malic acid was the least inhibitory, producing appreciable amylase when the initial pH was 2.0.

Salts and acids may slowly gain access to the embryo and affect its behavior during steeping. The presence of potassium bromate or Roccal in steep water considerably modifies the eventual behavior of the grain during malting.[2,5] The behavior is believed to be due to the effect of those substances on the embryo alone. The discovery by Paleg[16] that de-embryonated seed could be affected by GA has suggested that the response to GA-acids treatment described here may be attributed to their action on the endosperm.

Most of the organic acids used have been detected in cereal grains. Wall *et al.*[9] demonstrated that malic acid constituted 60% of the total organic acid of whole barley extract and that fumaric and malonic acids were present in significant quantity, but they detected only a trace of formic acid. Elliott[18] found significant quantity of malonic acid in germinated barley. It is known that malonic acid poisons the inter-conversions of succinate and fumarate in the Krebs cycle.[19] Fleming *et al.*[20] found that malonic acid effectively inhibited germination. Fumaric, malonic and malic acids were, however, least inhibitory in the acids employed in this experiment, which suggests that when concentration of these acids is controlled, they do not interfere with the development of alpha-amylase. Thus, GA stimulated development of alpha-amylase in the seed steeped in GA-malonic or -malic acids.

It is also possible that the acids play an important role in seed metabolism. The formation of lactic acid has been demonstrated in barley seedlings,[21] and its occurrence as a normal component of germinating barley was confirmed by Elliot[18]. Enders and Saji[22] demonstrated that the treatment with hydrogen peroxide alone at 0.5 or 0.1% in the presence of 0.1% lactic acid increased the germination of the grain and led eventually to a greater degree of proteolysis and improved modification in the grain. When lactic acid was used with GA on steeping, the acid was stimulatory at low concentrations, whose behavior was similar to that of formic acid.

On the basis of the experiment described above, it seems that the effects of acids on GA function to develop alpha-amylase in the seed are not dependent upon the pH value, but the kind and the concentration of acid employed for steeping the seed.

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