STARCH-LIQUEFYING ACTIVITY OF ALPHA-AMYLASE
I. Use of Pregelatinized Wheat Starch as Substrate[1]

Y. POMERANZ AND J. A. SHELLENBERGER

ABSTRACT

Starch liquefaction due to the action of malt, bacterial, and fungal alpha-amylases was studied. Pregelatinized wheat starch was used as substrate, and the Brabender Amylograph was employed to measure changes in viscosity resulting from amylolytic action. When compared on an equal basis of dextrinogenic activity, wheat-malt preparations exhibited highest, and fungal amylases lowest, liquefying abilities; bacterial alpha-amylases were comparable to wheat-malt. Comparison of a commercial fungal amylase with a pure crystalline alpha-amylase of fungal origin (Takadiastase A) showed that on an equal basis of dextrinogenic activity the preparations were equivalent in liquefying activity.

Amylolytic breakdown of the starch molecule involves liquefaction, dextrinization, and saccharification. Each of these phenomena has been

[1]Manuscript received August 14, 1961. Contribution No. 374, Department of Flour and Feed Milling Industries, Kansas State University, Manhattan, Kansas.

utilized to measure amylase activity: change in the viscosity of the substrate (starch paste), rate of dextrinization of soluble starch, and rate of maltose production during the initial phase of the enzyme action (8). The Wohlgemuth procedure (24), as modified by Sandstedt, Kneen, and Blish (21) to determine dextrinization by alpha-amylase, has achieved widespread popularity. In this method, alpha-amylase activity is expressed in terms of digestion time required for the enzyme to convert beta-limit dextrin containing active beta-amylase to products which give a red-brown coloration with iodine. The end point can be determined by use of a dextrin-iodine standard or permanent glass color standard (19).

The liquefying and dextrinizing functions of alpha-amylase are not always equivalent, so a knowledge of both activities is needed to study and to characterize more fully a given alpha-amylase (20). A number of methods (7,15,16) have been proposed to determine the liquefying properties of alpha-amylase. It is well established that bacterial alpha-amylase is most thermostable; cereal alpha-amylase possesses intermediate thermostability; and fungal alpha-amylase is relatively heat-labile. Consequently, the amylograph cannot be employed to evaluate the level of alpha-amylase of fungal origin added to wheat flour or native starch (18). This report deals with the liquefying action of bacterial, fungal, and malted-wheat alpha-amylases using pregelatinized wheat starch as substrate and employing the Brabender Amylograph to measure changes in viscosity resulting from amylolytic action.

Materials and Methods

Starch. Eleven samples of pregelatinized wheat starch were obtained from three manufacturers. Additionally, five samples of unmodified thick-boiling wheat starch were obtained from two manufacturers.

Amylase supplements. Four amylases used in this study were: (a) a commercial fungal amylase from *Aspergillus oryzae* having dextrinogenic activity of 14,500 SKB units per g.; (b) a commercial bacterial amylase from *Bacillus subtilis* having dextrinogenic activity of 10,500 SKB units per g.; (c) a partly purified wheat-malt alpha-amylase, having an activity of 500 SKB units per g.; and (d) a fungal alpha-amylase (Takadiastase A) lyophilized and thrice recrystallized from acetone. The crystalline enzyme was obtained from S. Matsubara. Its preparation, characteristics, and composition have been published (1,2,3,10, 13,17). Its dextrinogenic activity was 81,400 SKB units per g.

Each amylase was diluted with a 0.2% calcium chloride solution,

and added to the starch paste. In each case the dilution was prepared in such a way that 1 ml. of a 0.2% calcium chloride solution was added. Similarly, 1 ml. of the calcium chloride solution containing no enzyme supplement was added to the blank run. Assuming that up to ten calcium ions can be bound by a protein molecule of alpha-amylase, the amount of calcium added as calcium chloride represents at least a hundredfold molar excess of metal with respect to the microgram amounts of enzyme used in each determination (8).

Determination. Unless otherwise stated, 45 g. of starch were mixed for 2 minutes, by means of a manual egg beater, with 300 ml. of distilled water in a stainless-steel beaker. Three additional 50-ml. portions of water were used to rinse the beaker and the beater, and the starch suspensions and rinsings were placed in the bowl. The enzyme supplement was put directly into the amylograph bowl, between the first and second rinsings.

Discussions of the function, construction, and operation of the Brabender Amylograph have been published (4,6,22). Consequently, only modifications of the routine method, as employed here, are given in this paper. After the suspension was poured into the amylograph bowl, the kymograph was placed at a zero-time position, the contact thermometer adjusted to start heating at 25°C., and the instrument started. The temperature of the suspension was increased up to 75°C. in case of pregelatinized wheat starch and up to 95°C. in case of raw wheat starch at a constant rate of 1.5°C. per minute, as automatically provided by the instrument.

Results and Discussion

Results shown in Fig. 1 and Table I point to the fact that if fungal amylase is allowed to act on available (pregelatinized) starch, it is possible to employ the amylograph to study the liquefying action of the enzyme.

Whereas there is practically no difference between the liquefying action of bacterial or malt amylases on either raw or pregelatinized starch, the heat-labile fungal amylase seems to be inactivated prior to gelatinization and amylolytic susceptibility of the raw starch. Adding fungal amylase (up to 14 SKB units per 45 g. of starch) caused no detectable drop in viscosity, when tested by the normal amylograph procedure, employing native starch as substrate. When the native starch was gelatinized and cooled in the amylograph prior to addition of the enzyme, results similar to those obtained with commercial pregelatinized starch were recorded.

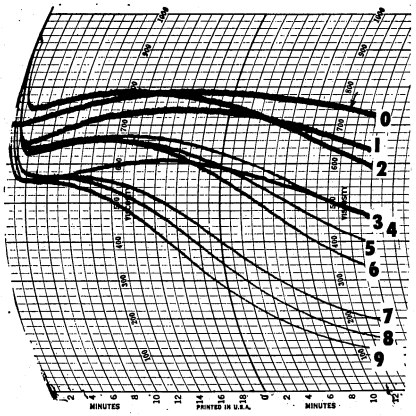

Fig. 1. Amylograms obtained on adding fungal alpha-amylase to pregelatinized starch (SKB units per 45 g. starch). 0 = control; 1 = 0.25; 2 = 0.50; 3 = 1.00; 4 = 2.00; 5 = 2.50; 6 = 3.50; 7 = 7.00; 8 = 10.5; 9 = 14.5 SKB units per 45 g. starch.

TABLE I

EFFECT OF AMYLASES ON LIQUEFACTION OF RAW AND PREGELATINIZED WHEAT STARCH
(Viscosity drop)

AMYLASE ORIGIN	RAW STARCH			PREGELATINIZED STARCH		
	Fungal	Bacterial	Wheat	Fungal	Bacterial	Malt
SKB units	*B.U.*	*B.U.*	*B.U.*	*B.U.*	*B.U.*	*B.U.*
0.5	10	270	320	110	300	350
1.0	20	400	390	180	400	450
2.5	0	530	540	335	560	600
7.0	10	600	590	530	630	640

The amylograms in Fig. 1 show the possibility of estimating, by the proposed method, levels of alpha-amylase ranging between 0.25 and 14.5 SKB units, and that there is a gradient of decrease in viscosity from graded additions of alpha-amylase. The viscosity at 71°C. was found, for a certain alpha-amylase level, to be reproducible within 40 B.U.

In Fig. 2 and Fig. 3 are given results of amylograms obtained with bacterial or wheat malt alpha-amylases. The shape of these curves is essentially the same as that in Fig. 1.

The amylograms in Fig. 4 were obtained from the action of graded increments of crystalline Takadiastase A added to pregelatinized starch.

Since major differences likely exist in the preparation of the starches by various manufacturers, differences in amylolytic susceptibilities of the starches were tested. The drop in viscosity due to amylolytic

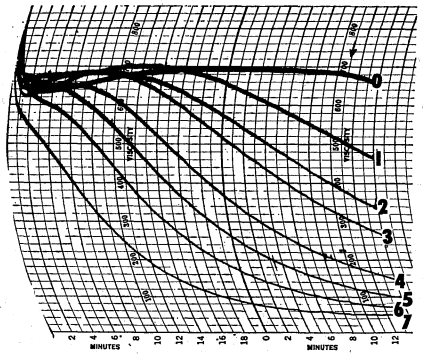

Fig. 2. Amylograms obtained on adding bacterial alpha-amylase to pregelatinized starch (SKB units per 45 g. starch). 0 = control; 1 = 0.25; 2 = 0.50; 3 = 1.00; 4 = 1.50; 5 = 2.50; 6 = 3.50; and 7 = 7.00.

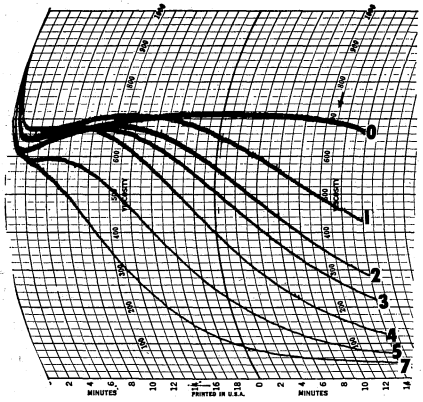

Fig. 3. Amylograms obtained on adding malt alpha-amylase to pregelatinized starch (SKB units per 45 g. starch). 0 = control; 1 = 0.25; 2 = 0.50; 3 = 1.00; 4 = 2.50; 5 = 3.50; and 7 = 7.00.

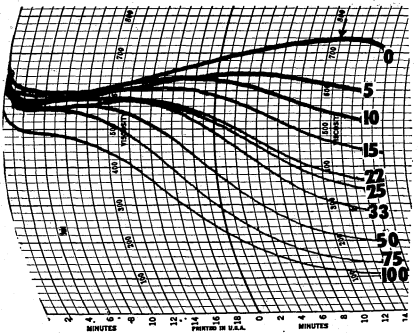

Fig. 4. Amylograms obtained on adding crystalline Takadiastase A to pregelatinized starch. The figures on the right side denote γ of preparation added to 45 g. of starch.

action on five different starch lots from one manufacturer was: (a) for adding 1.5 SKB units of fungal amylase, 230, 210, 140, 260, and 220 B.U.; (b) for adding 7.0 SKB units of fungal amylase, 480, 460, 330, 480, and 470 B.U. Testing four different starches from a second manufacturer, the following viscosity drops were recorded: (a) for adding 1.5 SKB units of fungal amylase, 120, 100, 140, and 80 B.U.; (b) for adding 7.0 SKB units, 500, 360, 430, and 400 B.U. The results indicate that a starch standardized specifically for the proposed method would be needed for comparisons among various laboratories.

Figures 5 and 6 show the results of drop in viscosity, under conditions of test, as correlated with the dextrinogenic activity of the various preparations. Note the wide difference in starch-liquefying properties of the preparations, compared on an equal SKB basis. Malt amylase

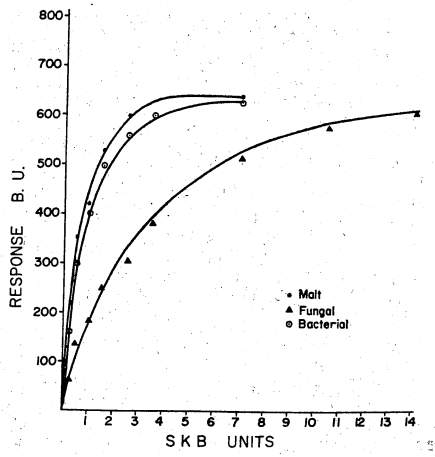

Fig. 5. Comparison between dextrinogenic and starch-liquefying properties of bacterial, fungal, and malt alpha-amylases.

was found to be far more active in the liquefying than in the dextrinizing properties; bacterial amylase was a close second. This confirms results of Redfern and Landis (20), who assessed the liquefying activity using a modification of the Jozsa and Johnston method, but is in disagreement with findings of Hollenbeck and Blish (12). Comparison of the crystalline Takadiastase A with the commercial fungal amylase, on an equal basis of dextrinogenic activity, shows them to be essentially equal in their liquefying activity. These results are summarized in Table II.

These results point to the fact that alpha-amylase in commercial preparations is the active enzyme involved in the liquefaction of starch

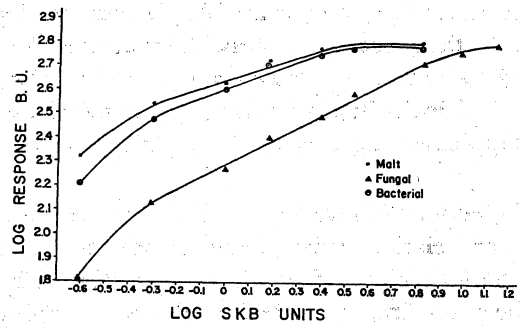

Fig. 6. Comparison between log of dextrinogenic and log of starch-liquefying properties of bacterial, fungal, and malt alpha-amylases.

TABLE II

COMPARISON OF LIQUEFYING ACTION OF COMMERCIAL FUNGAL AMYLASE WITH CRYSTALLINE TAKADIASTASE A
(Viscosity drop)

	Commercial Fungal Amylase	Crystalline Takadiastase
SKB units	*B.U.*	*B.U.*
0.40	80	120
0.81	160	200
1.20	250	270
1.62	290	320
2.02	340	370
2.67	390	435
4.07	480	530

gel, and that the liquefying action of a commercial preparation of fungal origin is essentially a measure of alpha-amylase activity.

Amylographs are being used by the cereal industry, under the assumption that the curves give an insight into the changes which starch undergoes within bread dough during the baking process. As the role of starch in bread quality has been demonstrated, attention has centered on the function of amylases during the actual baking period (23). If the production of sugar were the primary work of amylases, a baker should be able to produce high-quality bread by adding sugar. Such an addition has not been found to be a full substitute for amylase activity. There is strong evidence that a large portion of the total effect of the amylases on the starch granule actually takes place in the oven during the first stage of baking, through its effect on the gelatinization characteristics of the starch. Enzymatic digestion of starch during baking is limited by the concentration of amylases and their thermal inactivation temperatures; at the same time, a large

amount of the starch becomes available to enzyme degradation by gelatinization. It therefore seems feasible that the use of excess of available starch, acted on by enzymes under conditions resembling those in the baked loaf, should provide a reasonably good picture of the effect of supplementing bread dough with amylases from various sources.

Anker and Geddes (4) have found that for a series of unmodified starches a linear relation exists between logarithms of maximum viscosity and the logarithms of starch concentration. This linear relationship has been confirmed by Bechtel (5), who employed the Corn Industries Viscometer in his studies. The apparent viscosity, as measured by the amylograph, depends on several factors. In the case of tests made on raw starch, the height of the curve at maximum viscosity depends primarily on the extent of starch gelatinization which follows a first-order reaction; in the case of enzymatic hydrolysis and disintegration of the swollen granules, a second or higher order of reaction predominates (4). This might explain the fact that transformation of data to a logarithmic basis (see Fig. 6) resulted in a straight-line relationship only over a portion of the range of concentrations tested.

Published methods of differentiating between fungal and cereal amylase activities in wheat flour have been based on differences in thermal inactivation (9,14) or selective inactivation of cereal amylases on bentonite (11). Employment of the procedure proposed here permits distinguishing between fungal and either bacterial or cereal alpha-amylase. Whereas enzymes of either bacterial or cereal origin show a measurable amylolytic activity using raw starch as substrate, fungal amylase shows a measurable response in the case of pregelatinized starch only.

Acknowledgment

The authors gratefully acknowledge the aid of: L. Adler, Ministry of Trade and Industry, Haifa, Israel, who collaboratively tested the proposed procedure; J. Sullivan, who furnished a sample of purified malt; J. Fleming, who determined dextrinogenic activity; Shoji Matsubara, University of Osaka, who furnished a sample of Takadiastase A; and General Mills, Inc., Hercules Powder Company, Keever Starch Company, and Stein Hall and Company, Inc., who furnished samples of starch.

Literature Cited

1. AKABORI, S., and IKENAKA, T. Taka-amylase A. IV. The amino terminal amino acid of Taka-amylase A. J. Biochem. (Japan) **42:** 603–607 (1955).
2. AKABORI, S., IKENAKA, T., and HAGIKARA, B. Isolation of crystalline Taka-amylase A from Takadiastase Sankyo. J. Biochem. (Japan) **41:** 577–582 (1954).
3. AKABORI, S., IKENAKA, T., and OKADA, J. Taka-amylase A. II. Amino acid composition of Taka-amylase A. J. Biochem. (Japan) **41:** 803–813 (1954).
4. ANKER, C. A., and GEDDES, W. F. Gelatinization studies upon wheat and other starches with the amylograph. Cereal Chem. **21:** 335–360 (1944).

5. BECHTEL, W. G. A study of some paste characteristics of starches with the Corn Industries viscometer. Cereal Chem. **24:** 200–214 (1947).
6. BROWN, R. O., and HARREL, C. G. The use of the amylograph in the cereal laboratory. Cereal Chem. **21:** 360–369 (1944).
7. DEBECZE, G. I., VOTAW, J. W., and NANZ, R. H. Evaluation of diastatic activity of malt containing liquids by a modified Lasche test. Cereal Chem. **26:** 148–159 (1949).
8. FISCHER, E. H., and STEIN, E. A. Alpha-amylases. *In* The Enzymes, chap. 19, p. 325; Ed. P. D. Boyer, H. Lardy and K. Myrbäck, Vol. 9, 2nd ed., Academic Press, New York and London (1960).
9. FLEMING, J. R., MILLER, B. S., and JOHNSON, J. A. A method for the determination of relative amounts of malted-wheat, fungal (*Aspergillus oryzae*) and bacterial (*Bacillus subtilis*) alpha-amylase in mixture, and its application to malted wheat. Cereal Chem. **38:** 479–486 (1961).
10. HANABUSA, H., IKENAKA, T., and AKABORI, S. Taka-amylase A. III. Carbohydrate component in Taka-amylase A. J. Biochem. (Japan) **42:** 55–62 (1955).
11. HAYDEN, K. J. Improved method for detection and approximate estimation of fungal amylase supplementation of wheat flour. J. Sci. Food Agr. **12:** 123–127 (1961).
12. HOLLENBECK, C. M., and BLISH, M. J. Parallelism between starch dextrinizing and liquefying activities of amylases. Cereal Chem. **18:** 754–771 (1941).
13. IKENAKA, T. Taka-amylase A. V. Carboxyl terminal group of Taka-amylase A. J. Biochem. (Japan) **43:** 255–261 (1956).
14. KNIGHT, R. A. Detection and estimation of fungal amylases in flour. J. Sci. Food Agr. **11:** 54–60 (1960).
15. LANDIS, Q., and REDFERN, S. Methods for determination of alpha-amylase. III. Improved starch-liquefying method. Cereal Chem. **24:** 157–167 (1947).
16. LOHMAR, R. L., WEAKLEY, F. B., and LAUTERBACH, G. E. Controlled degradation of waxy corn starch by malt alpha-amylase. Cereal Chem. **33:** 198–206 (1956).
17. MATSUBARA, S., IKENAKA, T., and AKABORI, S. Taka-amylase A. VI. Alpha-maltosidase activity of Taka-amylase A. J. Biochem. (Tokyo) **46:** 425–431 (1959).
18. MILLER, B. S., and JOHNSON, J. A. Fungal enzymes in baking. Baker's Digest **29:** 95–100 (1955).
19. REDFERN, S. Methods for determination of alpha-amylase. IV. A glass end point color standard for use in the dextrinizing method; effect of temperature and starch lot on this method. Cereal Chem. **24:** 259–267 (1947).
20. REDFERN, S., and LANDIS, Q. Methods for determination of alpha-amylase. II. Liquefying and dextrinizing activities of alpha-amylases from different sources. Cereal Chem. **23:** 1–15 (1946).
21. SANDSTEDT, R. M., KNEEN, E., and BLISH, M. J. A standardized Wohlgemuth procedure for alpha-amylase activity. Cereal Chem. **16:** 712–723 (1939).
22. SELMAN, R. W., and SUMNER, R. J. The use of the amylograph for flour malt control. Cereal Chem. **24:** 291–299 (1947).
23. WALDEN, C. C. The action of wheat amylases on starch under conditions of time and temperature as they exist during baking. Cereal Chem. **32:** 421–431 (1955).
24. WOHLGEMUTH, J. Über eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments. Biochem. Z. **9:** 1–9 (1908).