Fungus and Enzyme Preparation of Aspergillus Oryzae Amylase and its Use

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(Received September 26, 1951)

Introduction

In 1934, the author[1] discussed the relation between various chemicals and fungi, and pointed out that thiourea exhibits a definite toxic value for fungi combined with neutral and harmless properties for human tissue. Application of this finding on the preparation or food and its value to fruits has aroused great interest which has appeared in recent literatures.[2],[3],[4]

The present paper of this series of investigations deals chiefly with a practical application of fungal amylase to the textile industry.

The fungal amylase taka-diastase[5] is a group of a great many enzymes among which protease, invertase and a few others are important ones. The mixture of such enzyme gives an undesirable result in one case but in an other case it gives a much better result than when used as a relatively pure amylase. The former case is seen in bread making[6] and the latter in desizing textiles in which the starch for sizing is not always pure.

Therefore, it is desirable to know how much of these enzymes are present in the amylase preparation before they are used in practice.

1) Hydrolysis of starch.—Saccharogenic hydrolysis of Lintner's starch by taka-diastase is found roughly to be a zero order of reaction since the value of n in the equation $\frac{dx}{dt}=K(a-x)^n$ is found to be -0.34.[7]

2) Estimation of Invertase.—To estimate this enzyme the author described the method[8] in which the amount of glucose is present and convenient for its calculation in accordance with the number of titer cc. which is not exactly reciprocally proportional to the true amount of produced sugar. This amount can now be calculated by the following equation with a fairly experimental agreement.

$$X=\left(0.5000\div\frac{Y}{5}\right)+\frac{0.5000(Y-5)}{1000}$$

X=glucose % in the case of 0.1-0.5% titer solution, Y=titer cc.

3) Use in Banana Powder Making.—In bread making, from two to three percent of sucrose is usually added to the flour for baking bread leavened with yeast which contains a considerable amount of invertase compared with that in the amylase preparation. Therefore, when a relatively small amount of the preparation is added to the dough, the invertase itself might not affect the

(1) T. Harada, This Bulletin, **9**, 186 (1934).
(2) Denny, *Contrib. Boyce Thompson Inst.*, **7**, 55 (1935); *C. A.*, **29**, 6966 (1935).
(3) Childs and Siegler, *Science*, **102**, 68 (1945); *Ind. Eng. Chem.*, **38**, 82 (1946).
(4) Hartzell, *Contrib. Boyce Thompson Inst.*, **12**, 472 (1942); also refer Reineke and Turner, *Poultry Sc.*, **24**, 340 (1945); *C. A.*, **39**, 4968 (1945).
(5) The amylase preparation from pure culture on bran does not show the presence of alcohol oxidizing enzyme.
(6) T. Harada, This Bulletin, **24**, 105 (1951); *J. S. R. I.*, **44**, 189 (1950).
(7) This value was formally represented by the author as 2.34 and it was, therefore, thought that the reaction is to be a bimolecular one *Ind. Eng. Chem.*, **22**, 1424(1931).
(8) T. Harada, *Ind. Eng. Chem., Anal. Ed.*, **3**, 1 (1931).

Table 1

Titer cc. and Glucose %

No.	Titer, cc.	Glucose % obtained	Glucose % calculated	Error
1	5.00	0.5000	0.5000	0
2	6.25	0.4000	0.4000	0
3	7.50	0.3380	0.3346	0.1
4	8.50	0.3000	0.2959	0.1
5	10.00	0.2550	0.2525	1.4
6	13.00	0.2000	0.1986	1.0
7	17.50	0.1500	0.1492	0.5
8	21.75	0.1250	0.1234	1.5
9	29.00	0.1000	0.0982	1.8

bread compared with the control. However, in the banana powder making industry, the preparation helps a great deal to give a more digestive and nutritious product since a considerable amount of sucrose and the amylase preparation are both used. The analytical result was made by the author for one of the samples prepared by North-John's company, U. S. A. It is given in Table 2.

Table 2

Analysis of the banana powder obtained by treating with amylase and the control

	Untreated, %	Treated, %
Reducing sugar as glucose	28.14	43.84
Sucrose	52.00	41.23
Starch	7.68	3.53
Total carbohydrate	90.96	90.96

4) Effect of Electrolyte upon the Liquefaction of Starch Paste by Amylase.—The action of fungal amylase upon a swollen starch or starch paste is presumed to take three steps; that is to say, the starch is first transferred into soluble starch, then to dextrine and finally to sugar. The ratio of liquefying power to saccharogenic power differs depending on the origin of amylase and the process of the preparation and the condition.

The viscosity of starch paste in enzyme treatment is greatly influenced by the addition of an electrolyte. Among the electrolytes, ammonium, sodium and potassium chlorides, ammonium sulphate, potassium bromide and iodide, have the greatest effect, and these are followed in order by ammonium sulphate, potassium chloride, potassium bromide and iodide respectively when they are used by percent in weight; that is $NaCl \fallingdotseq NH_4Cl > (NH_4)_2SO_4 > KCl > KBr > KI$.

The activating power of the enzyme as measured by the liquefaction of starch is quickly established with a trace of the electrolyte NaCl in the case of pancreatic amylase, the maximum being obtained with a concentration of approximately 0.0025-0.0100 percent in the buffered starch solution (200 cc. with 50 cc. of 0.2 M H_2KPO_4 and 17.80 cc. of 0.2 M NaOH) to *p*H 6.6, and then is slowly decreased as the concentration increases. This buffer istelf decreases the viscosity of starch paste solution about 8.56 percent. The experimental results are shown in Tables 4, 5, 6, 7 and 8.

It appears that a trace of NaCl is essential for the liquefaction of starch by pancreatic amylase; in other words, the enzyme can not be liquefied without NaCl. The small decreases (0.010) in the viscosity is due probable to an experimental error in this type of experiment. In this, it differs from malt and fungal amylases. The last named two enzymes are not essential for liquefying action but are greatly activated by the salt.

The decrease of viscosity of starch paste by electrolyte alone is cosiderable. The action of electrolyte on starch is not clearly known. There is a lack of information regarding the effect of electrolyte on the viscosity of solution or paste of starch. In this connection, Boutaric and Chapeaux's report[9] appeared recently in the chemical abstract; however, unfortunately neither the details nor the original paper of the above authors are available to the author at present in Japan.

As the result of experiment, it was found that the viscosity is not alone due to the degree of acidity of the solution, since the chloride of the alkaline earth metal $CaCl_2$ liquefies much more than that of $AlCl_3$. Among phosphates, the acid one gives a greater effect than that of the alkaline one. Moreover (addition of 5 cc. of 0.2 M) sodium hydroxide in many experiments gives only a little change on the liquefaction of starch paste on standing at room temperatures.

5) Viscosity of Native Starch Solution and Time of Heating at 50°C.—The viscosity of the solution of starch paste, relative to that of water is calculated by means of the formula:

$$\eta\ (\text{starch solution}) = \frac{(\text{time} \times \text{density})_{\text{starch}}}{(\text{time} \times \text{density})_{\text{water}}}$$

The viscosity of 0.5 percent native potato starch solution was little influenced (not more than 5 percent) by heating at 50°C. for more than one hour.

As experiment, five grams of native potato starch were introduced into a large beaker with a small amount of water (about 30 cc.) to which about

Table 3

Relation between the Viscosity of 0.5 percent Native Starch Solution and Time of Incubation at 50°C. (viscosity relative to that of water at 25°C.)

No.	1	2	3	4
Time in min.	0	5	10	15
η change	2.4375	2.5000	2.4375	2.5000

No.	5	6	7
Time in min.	20	25	30
η change	2.4375	2.4375	2.4375

(9) Boutaric and Chapeaux, *Compt. rend.* **214**, 949 (1942); *C. A.*, **39**, 236 (1945).

Table 4

Influence of NaCl on Starch Liquefying Action of Fungal Amylase (Viscosity relative to that of water at 25°C.) with 0.0050% Solution

No.	A		B	C	D	E	F
	NaCl %	pH	η controls water +NaCl +NaOH +enzy.	η controls starch soln. +NaCl +NaOH +enzy.	η starch soln. +NaCl +enzy. then add. NaOH	η reduced, due to NaCl (C_1–C_n)	η actively reduced by enzy. (C_1–D_n)
1	—	6.2	1.0002	2.3134	2.0004	—	0.3130
2	0.0050	6.2	1.0002	2.2509	1.6257	0.0625	0.6877
3	0.0100	6.2	1.0315	2.1884	1.4378	0.1250	0.8756
4	0.0200	6.2	1.0327	1.9383	1.3755	0.3751	0.9379
5	0.2000	6.2	1.0628	1.6884	1.1882	0.6250	1.1252
6	0.5000	6.2	1.0632	1.5012	1.1259	0.8122	1.1875

No.	G	H	I	J	K	L
	η reduced, due to enzy. (C–D)	η starch soln. (C–B)	η reduced, due to NaCl and enzy. combined (D_1–D_n)	η starch soln. alone by enzy. (D–B)	η reduced, due to enzy. activation by NaCl (H_1–H_n)	η corresp. soluble starch soln. (0.5%)
1	0.3130	1.3132	—	1.0002	—	1.0627
2	0.6252	1.2507	0.3747	0.6255	0.0625	1.0627
3	0.7506	1.1569	0.5626	0.4063	0.1563	1.0627
4	0.5628	0.9056	0.8754	0.3428	0.4076	1.0638
5	0.5002	0.6252	1.0627	0.1254	0.6880	1.0630
6	0.3753	0.4380	1.1250	0.0627	0.8752	1.0634

Table 5

Influence of NaCl on Starch Liquefying Action of four different Amylase Preparations (viscosity relative to that of water at 25°C.) with 0.0050% Solution

P. D. = pancreatic diastase, T. D. = taka-diastase, M. D. = malt diastase, B. D. = bacterial diastase (Minagawa's commercial, is mixed with salt ?).

No.		P. D.	T. D. No. 1	T. D. No. 2	M. D.	B. D.*
	NaCl %	η decreased, due to enzy.	η decreased, due to enzy. (L. V. 3000)	η decreased, due to enzy. (L. V. 2030)	η decreased, due to enzy. (L. V. 780)	η decreased, due to enzy. (L. V. 570)
1	—	0.010	0.313	0.188	0.063	0.813
2	0.0025	1.576	—	—	—	—
3	0.0050	1.675	0.625	0.625	0.438	0.813
4	0.0100	1.513	0.751	0.751	0.469	0.743
5	0.0200	—	0.563	0.563	0.375	0.532
6	0.1000	1.274	—	—	—	—
7	0.2000	—	0.500	0.500	0.250	0.313
8	0.2500	1.183	—	—	—	—
9	0.5000	0.978	0.375	0.375	0.156	0.219

* The author thanks Dr. T. Minagawa for his bacterial amylase which was kindly given for this experiment.

300 cc. of boiling distilled water was added with a moderate constant stirring by means of a glass rod. The solution was boiled by heating for about ten minutes or over, cooled, then transferred to a 500 cc. volumetric flask and then diluted to the mark with freshly boiled and cooled distilled water.

A series of glass-stoppered Erlenmeyer's flasks containing 50 cc. of the solution of starch were incubated in a 50°C. thermostat for about 30 minutes until the internal temperature reached the temperature of the thermostat.

Each of the seven solutions was treated further for zero, 5, 10, 15, 20, 25 and 30 minutes respectively, and exactly at the end of the time of incubation, the solutions were treated with 5 cc. of 0.2 M NaOH and of water and mixed.

The rate of flow and the density of the respective solutions were determined by means of an Ostwald's viscosimeter and a picnometer at 25°C. respectively.

6) Method of Measuring the Liquefaction of

Starch Paste by Amylase.—150 cc. of the 0.5 percent starch solution prepared as above in (5) was transferred by means of a pipette very carefully free from saliva into several 250 cc. glass-stoppered Erlenmeyer's flasks containing respectively 0.0038, 0.0075, 0.0150, 0.1500, 0.3750 and 0.7500 g. of sodium chloride so as to give the solutions of 0.0025, 0.0050, 0.0100, 0.2500 and 0.5000 percent as the examples.

Two series of 100 cc. capacity of Erlenmeyer's flasks with glass stoppers, each containing 50 cc. of starch solution as above described, were prepared by means of a thoroughly cleaned 50 cc. pipette. The series of flasks were placed in a 50°C. thermostat for about 30 minutes and then they were treated as follows: Five cc. of a clear enzyme solution (0.005 percent) was added by means of a 5 cc. pipette to each solution and allowed to stand for exactly 5 or 7 minutes according to the strength of the enzyme preparation. At the end of this time 5 cc. of 0.2 M NaOH solution was added to destroy the activity of the enzyme. However, for the controls the NaOH solution was first added and then the enzyme solution followed. The viscosity of the each solution was determined as described above.

In the experiment, the substrate starch solution for liquefying power test of amylase was not buffered except in the case of pancreatic enzyme, since it has little meaning in the textile desizing factory, or is rather a contamination. The results are given in the following tables which are the average values of at least three experiments.

7) **Desizing of Cotton.**—The wetting agent Nekal BX, as the example, gives great help for desizing of cotton cloth when it is added to the enzyme solution. However, when the cotton material is, previously, treated with boiling water, the agent gives little help for the purpose of desizing. In practiec in the factory, it is suggested that the sized material be first passed through a boiling NaCl or other salt solution and then through the enzyme solution containing NaCl or other salt at the temperature of 45-50°C. This is the conclusion of many desizing experiments.

The order of salts with respect to decreasing the viscosity of starch solution was $CaCl_2 > NaCl > AlCl_3 \fallingdotseq KH_2PO_4 \fallingdotseq KCl > Na_2HPO_4$.

8) **Making of "Ame".**—Fungal amylase preparation gave a slightly colored product, to "ame", a favorite kind of Japanese candy, when potato starch paste was treated with the amylase in the usual process; while malt amylase preparation gave a much better white product.

Summary

The liquefaction of starch by means of electrolytes and of amylase preparations of various origins has been studied by the viscosity method. It appears that the presence of NaCl is necessary for the liquefaction of starch paste by pancreatic one. Nevertheless, for other enzymes, NaCl is not essential for the liquefaction but the presence of NaCl accelerates greatly the enzymatic action.

The maximum activation appears at about 0.01 percent cocentration. The relation between various salts and viscosity of starch solutiqn has been also studied.

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Table 6

Influence of NaCl on Starch Liquefying Action of Two different Amylase Preparations (viscosity relative to that of water at 25°C.) with 0.0100% Solution.

No.	NaCl %	T.D. No. 1 η decreased, due to enzy. G (C-D)		η decreased, due to enzy. M. D. G (C-D)	
1	—	0.3755	opaque	0.2505	
2	0.0050	0.8131	opaque	0.5631	
3	0.0100	1.0632	trans-parent	0.6250	opaque
4	0.0200	0.8755	trans-parent	0.5004	opaque
5	0.2000	0.6254	trans-parent	0.3127	opaque
6	0.5000	0.4378	trans-parent	0.2502	opaque

Table 7

Influence of various Salts on Starch Liquefying Action of Taka-diastase Preparation (viscosity relative to that of water at 25°C.)

No.	Salt 0.0100%	η obs.	η reduced by enzy.	order of salts
1	NaCl	1.4065	0.9069	$NaCl \fallingdotseq NH_4Cl$
2	NH_4Cl	1.4065	0.9069	$(NH_4)_2SO_4 > KCl >$
3	$(NH_4)_2SO_4$	1.4375	0.8759	$KBr > KI$
4	KCl	1.4692	0.8442	
5	KBr	1.5945	0.7189	
6	KI	1.7201	0.5933	

Table 8

Relation between Salt and Viscosity of Starch Solution at 25°C.

No.	Salt 0.2000%	pH	η obs.	η reduced by salt
1	NaCl	6.2	1.5764	0.7882
2	$CaCl_2$	6.4	1.5461	0.8185
3	$AlCl_3$	4.8	1.6067	0.7579
4	KH_2PO_4	5.0	1.6067	0.7579
5	KCl	6.2	1.6067	0.7579
6	Na_2HPO_4	7.8	1.6370	0.7276