oxidized and that they varied slightly as to the rate with which oxidation proceeded; hence, any permanganate method for their accurate determination must depend upon definitely fixed conditions.

Likewise, it was obvious that commercial creosote and guaiacol could not be determined by this procedure, since they are mixtures of several more or less related phenols that are not present in like proportion in different specimens. However, with single solutions of several common phenols and closely related compounds, fairly gratifying results were obtained. Pyrogallol, pyrocatechin, resorcinol and hydroquinone, from all of which the CH_3 group is absent, were readily and completely oxidized.

Benzoic acid was very slightly attacked while under similar conditions salicylic acid and salol were completely oxidized. Thus it would seem that the phenolic OH group predisposes towards a complete oxidation and that many uninvestigated phenols and closely related compounds would give analogous reactions. In making up solutions of the several phenols, sufficient $\text{N}/2$ NaOH was added when necessary, to insure ready solution.

The following table is self-explanatory:

SUBSTANCE	PER-CENTAGE FOUND	SUBSTANCE	PER-CENTAGE FOUND
Pyrogallol.....	{ 100.4	Hydroquinone.....	{ 99.49
	{ 100.1		{ 99.56
Pyrocatechin.....	{ 100.2	Salicylic acid.....	{ 99.79
	{ 99.9		{ 100.2
Resorcinol.....	{ 100.5	Salol.....	{ 99.77
	{ 100.4		{ 99.91

The alkaline permanganate method is especially applicable for the quantitative estimation of the above compounds when they occur individually in very small amounts in single solutions or in conjunction with substances not readily oxidized.

DEPARTMENT OF CHEMICAL RESEARCH
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SOME PROPERTIES OF KOJI-DIASTASE

By G. KITA

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The properties of the saccharifying enzymes of koji (a culture of *aspergillus oryzae* on steamed rice) have been studied by many investigators and are supposed to be comparatively well understood. There are, however, certain obscurities which exist in regard to the identification and classification of these enzymes. They must be cleared up on account of their scientific and practical importance.

I have lately been occupied in a series of investigations relative to Soya or Shoyu (Japanese sauce fermented from beans, wheat and salt) making, and incidentally cleared up certain of the above points.

1. IS SACCHARIFICATION OF STARCH BY KOJI-DIASTASE CARRIED OUT BY TWO DIFFERENT ENZYMES, DIASTASE (AMYLASE) AND GLUCASE, SUCCESSIVELY?

It has been generally thought that koji contains two kinds of saccharifying enzymes, *viz.*, diastase (amylase) and glucase, and it is supposed that the glucose present in a liquid saccharified by means of

koji is produced by the two enzymes above mentioned. It has been affirmed by some investigators that while the glucase present in koji does not act rapidly, yet, the sugar finally produced in the saccharification of starchy matters with koji is invariably glucose, and that maltose is present only in a negligible quantity. Evidence in support of the above assertions is still meager and I was led to think that there may be present in koji another new enzyme, which is altogether different from glucase, and which can convert starch directly to glucose without intermediate steps. The following experiments were made:

Experiment 1.—To each 200 cc. of a 2 per cent. solution of maltose (moisture = 4.25 per cent.) and also starch (moisture = 16.99 per cent.), 20 cc. of the solution of enzymes (corresponding to 2 grams of koji) were added. After allowing the enzymes to act for three hours at 50° C., the reducing action of the digested liquid on Fehling's solution was tested and a rough estimation of glucose was made by means of the glucosazone reaction.

In the determination of the osazone, 100 cc. of each liquid were boiled 1½ hours with 4 grams of phenylhydrazine hydrochloride and 6 grams of sodium acetate, filtered and washed with 100 cc. boiling water. This, of course, cannot be said to be a perfect method either for the isolation or determination of glucosazone, but is sufficiently accurate for comparison of results.

Solution acted upon by enzymes	Starch	Maltose
Cc. saccharified solution required to reduce 50 cc. Fehling's solution.....	18.6	21.0
Grams of glucosazone obtained from 100 cc. of saccharified solution.....	0.2575	0.1164

Experiment 2.—A like experiment was performed with Taka-diastrase. In this case each 100 cc. of a 2 per cent. solution of maltose and starch, respectively, was hydrolyzed with 20 cc. of a 1 per cent. solution of the enzyme. For the determination of glucosazone 50 cc. of each solution were taken:

Kind of solution acted upon by enzymes	Starch	Maltose
Cc. saccharified solution required to reduce 50 cc. Fehling's solution.....	30.0	23.6
Grams of glucosazone obtained from 100 cc. of saccharified solution.....	0.2088	0.0772

As will be seen in the above experiment, the reducing power of the starch solution after digestion is so strong that we can hardly compare the rate of hydrolysis of maltose to that of starch. Moreover, the quantity of glucosazone from the starch solution is very much higher. The presence of dextrine in the production of glucosazone does not seem to possess remarkable influence, but the presence of maltose has a tendency to give an increased quantity of glucosazone when phenylhydrazine hydrochloride is used in excess. Therefore, in the hydrolysis of starch by means of koji-diastrase, it is evident that glucose is not produced principally by the action of glucase, but directly without the aid of it. Dr. Nishisaki¹ asserts that in the case of saccharifying starch by means of koji, the sugar produced thereby is composed in its first stage, of maltose, on account of the weak activity

¹ J. Chem. Soc., Tokyo, 29, 325.

of glucase. But this must have arisen from an error because of his presumption of the presence of malt-diastase in koji.

II. INFLUENCE OF SALT ON ENZYMS IN KOJI

It is a well-known fact that enzymic activity is lost by heating, but so far as I know the effect of heating it in the presence of salt has not yet been investigated. In the soya mash, enzymes act in salt solution and it is, therefore, necessary to find out the relation of enzymes toward heat in the presence of salt. With this object in view, I made the following experiments which have fortunately proved that NaCl has a protecting influence on koji-diastase but not on malt-diastase; while Na_2HPO_4 , asparagin, and H_2SO_4 have an injurious effect even on koji-diastase.

Experiment 3.—One hundred cc. of a 2 per cent. starch solution, taken in each of three different vessels, were digested for two hours at 50°C . with 5 cc. solution of enzymes (100 grams koji or malt macerated in 1,000 cc. water for 10 hours), into two of which 20 grams of NaCl were added in two ways: one from the beginning and the other after digesting for three hours. The remaining one was kept blank for control.

NO. OF CC. OF THE SACCHARIFIED SOLUTION USED FOR THE REDUCTION OF 50 CC. FEHLING'S SOLUTION

No.	Kind of digestion	Enzymic solution from koji	Dried malt	Green malt
1	Not heated.....	50.2	41.4	25.2
2	Heated 3 hrs. at 50°C . and added NaCl.....	67.6	90.0	25.2
3	Heated 3 hrs. at 50°C . after addition of NaCl.....	55.6	200.0	57.4

Experiment 4.—The experiment was carried as in Experiment 3, except that Na_2HPO_4 , asparagine and H_2SO_4 were used instead of NaCl. For 100 cc. koji extract, 5 grams Na_2HPO_4 , 5 grams asparagine or 14 cc. of 1 per cent. H_2SO_4 solution were added.

NO. OF CC. OF THE SACCHARIFIED SOLUTION USED FOR THE REDUCTION OF 50 CC. FEHLING'S SOLUTION

No.	Kind of digestion	Enzymic solution with asparagine	Na_2HPO_4	H_2SO_4
1	Not heated.....	35.2	57.0	87.5
2	Heated before addition.....	44.5	65.4	100.0
3	Heated after addition.....	92.5	112.4	140.0

III. THE INFLUENCE OF SALT ON SACCHARIFYING ACTIVITY OF KOJI-DIASTASE

The results obtained by previous investigators with regard to the influence of salt, acid, etc., on the saccharification by malt- and koji-diastase agree on the point that a very small quantity of these substances acts favorably, but that increase of the same is injurious.

Among various authors, Dr. Saito¹ says that the activity of koji-diastase is injured by the presence of salt, for instance, in a ten per cent. salt solution its activity is lessened to 11.3% and in twenty per cent. solution to 7.6 per cent. S. Mori,² however, says that a large quantity of NaCl gives no inhibitory influence on koji, but he attributed this observed influence to a peculiarity of some kinds of koji. His result is inaccurate since a development of organisms took

place in his experiment. At that time, I also observed the similar curious phenomenon which led me at once to entertain an opinion like Mori. Men employed in soya-making alleged that in practice where a considerable quantity of salt is present in mash, no retarding of enzymic action is noticed. Such diversity of opinion was found lately to have originated in the fact that when the enzymic solution is concentrated a large quantity of sodium chloride does not apparently exert an inhibitory influence, while in a less concentrated enzymic solution even a smaller quantity of salt will have a very injurious action. In general, the influence of salt on diastase is related to the concentration of the enzym solution. The following experiments show this relation clearly:

Experiment 5.—One hundred cc. of a 20 per cent. soluble starch solution with or without 40 grams NaCl were saccharified with 100 cc. koji extract (200 grams wheat koji extracted in 1000 cc. water) and were filtered after 16 hours standing at 50°C . The iodine reaction of the solution without salt was purple or light red after 10 or 30 min., respectively, while that of the salt-containing solution was somewhat deeper in color. After 30 min. 5 cc. concentrated caustic alkali solution were added in order to inhibit further diastatic action and then sugar was determined after diluting the solutions to five times their own volume. To the digestion which did not contain NaCl the same amount of NaCl was added as was in the other, so that the conditions might be similar in conducting the sugar determinations.

NO. CC. OF THE DILUTED SUGAR SOLUTION TO REDUCE 25 CC. FEHLING'S SOLUTION

Digestion with NaCl	Digestion without NaCl
16.5	15.0

Experiment 6.—One hundred cc. of a 2 per cent. or 20 per cent. soluble starch solution were saccharified with 10 cc. of the koji extract at 50°C . wherein 20 grams NaCl were added before or after digestion. After the digestion was over, NaCl was added to the one which did not contain the same, as was done in the previous experiment.

The reaction of iodine toward these liquids was tested and the following result was obtained:

Duration of digestion in minutes	2 per cent. starch solution		20 per cent. starch solution	
	Digestion with NaCl	Digestion without NaCl	Digestion with NaCl	Digestion without NaCl
20	purple	red	blue	blue
30	red	red	blue	blue
60			purple	purple
120			red	red

Similar digestion was repeated after 30 minutes; 5 cc. caustic alkali solution were added in one case, and in the other after 120 minutes, so that further diastatic action is entirely arrested. Then, diluting the digestion in case of 30 minutes duration to five times and in case of 120 minutes duration to ten times its original volume, it was tested how many cc. of such dilution can completely reduce 25 cc. of Fehling's solution.

¹ *Centrbl. Bakt., II Abt.*, **17**, 102.

² *J. Pharm. Soc., Tokyo*, **311**, 1-10.

¹ *J. Chem. Soc., Tokyo*, **13**, No. 145, pp. 221-235.

No. cc. OF THE DIGESTION TO REDUCE 25 CC. FEHLING'S SOLUTION			
30 minutes digestion		120 minutes digestion	
with NaCl	without NaCl	with NaCl	without NaCl
23.2	17.5	45.5	30.3

Experiment 7.—Two grams soluble starch in 500 cc. water with different quantities of NaCl were saccharified with 10 cc. koji extract at 50° C. After 30 min., 10 cc. alkali solution and NaCl were added so that each solution contained 100 grams NaCl in total solution.

REACTIONS TOWARD IODINE			
No. of solution	No. of grams of NaCl added before reaction	After 10 min.	After 30 min.
1	0	red	red
2	20	purple	red
3	100	purple	purple

No. of solution	No. cc. of sugar solution to reduce 5 cc. Fehling's solution
1	17.0
2	26.0
3	45.0

In Nos. 5 and 6 we see that the influence of NaCl depends upon the concentration and not on the ratio of starch to the enzymic quantity. The more dilute is an enzymic solution the stronger is the inhibitory action of sodium chloride.

From Exps. 6 and 7 it is clear that the inhibitory action of NaCl does not relate to dilution when the proportion of NaCl to enzym is constant.

IV. CAN KOJI-DIASTASE BE PRESERVED IN BRINE?

The stability of the koji-diastrase in brine is not yet well known and it is a very important factor in soya making, where a long period of activity is necessarily maintained. I have kept diastase in brine for a month in summer and compared it with that kept in a dried condition in a desiccator. The result was as follows:

Experiment 8.—One hundred cc. of a 2 percent. starch solution was saccharified for 3.5 hours at 50° C. with an enzymic solution (1 gram Taka-diastrase, 15 grams NaCl in 100 cc. water), and its reducing power was determined.

No.	Preservation of Diastase	No. cc. of saccharified solution to reduce 25 cc. Fehling's solution
1	In brine.....	30.8
2	In dry condition with envelope of black glazed paper	28.3
3	In dry condition in thin layer exposed to sunlight....	33.4

It will be seen from these results that the diastatic power of the enzym in brine was stronger than that which was exposed to sunlight. The gelatine liquifying power of the same diastase was in the same order. From this and other data I have, I believe the strong diastatic power exerted in the old mash which was kept about a year was principally due to the koji originally used.

SUMMARY

I. Though koji may contain two different saccharifying enzymes, *viz.*, amylase and glucase, the total quantity of the glucose in a saccharified solution could not be produced from maltose by the action of glucase alone; hence, koji must contain some

other kind of diastase which produces glucose directly from starch without the aid of glucase.

II. Common salt has a protecting action on koji-diastrase under heating but not on malt-diastrase, while Na₂HPO₄, asparagine and H₂SO₄ impair its activity more quickly.

III. The inhibitory action of salt on koji-diastrase has a certain relation to the concentration of diastase. In a dilute enzymic solution it is very strong but not in a concentrated enzymic solution. It is therefore necessary in every case to note concentration of diastase when we consider the influence of some salt upon it.

IV. The activity of koji-diastrase is conserved in brine for a long period.

DEPARTMENT APPLIED CHEMISTRY
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APPLICATION OF THE AMMONIUM CARBONATE METHOD FOR THE DETERMINATION OF HUMUS TO HAWAIIAN SOILS¹

By J. B. RATHER

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The ammonium carbonate method for the determination of humus in soils² has been used on a number of samples of Texas soils, of different types met with in the United States, including the Travis, Orangeburg Houston, Laredo, Norfolk, Lufkin and Cameron series, and in every case the results have been entirely satisfactory, the "ash" averaging less than 0.40 per cent. While this method has proved to be reliable in this laboratory, it has been shown by Kelley and McGeorge³ that on certain soils of Hawaii the method does not give good results. This is due to the nature of the clay and not to the amount of clay present, for we have precipitated the clay completely in a solution containing 33.45 per cent. "ash" as determined by the official method.

Kelley and McGeorge modified the ammonium carbonate method by adding 4 grams ammonium carbonate per hundred cubic centimeters instead of 0.5 gram. This coagulated the clay in most cases, but they claim that the organic matter was partially precipitated.

These investigators propose a method for the removal of the clay by filtration by means of reduced pressure through a Pasteur-Chamberland tube and washing with ammonia as was done by Alway, *et al.*⁴ In connection with this it might be of interest to state that the filtration of humus solutions by reduced pressure has been done by Fraps and Hamner,⁵ who themselves do not claim originality for the process. Fraps and Hamner state that the method is very tedious and that organic matter may not pass through the filter. Neither Cameron, who first used the porcelain tube, Fraps and Hamner, nor Alway, *et al.*, recommended the method.

¹ Under the general direction of G. S. Fraps, chemist. Read at the meeting of the Association of Official Agricultural Chemists, Washington, D. C., September, 1912.

² THIS JOURNAL, 3, 660.

³ *Ibid.*, 4, 9, 604.

⁴ Nebraska Exp. Sta., Bull. 115.

⁵ Texas Exp. Sta., Bull. 129.