BIOCHEMICAL STUDIES ON "SOTETSU" (*Cycas revoluta* Thunb.). IV. ON SOTETSU-EMULSIN.

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Dr. K. Yoshimura[1] in our college discovered that "Sotetsu," especially its seed, contains a fair amount of formaldehyde, and stated that the sotetsu-poison is due to the presence of the formaldehyde. He prepared a distillate from the alcoholic extract of mashed sotetsu-seed, in which he detected formaldehyde by using Nessler's reagent and ammoniacal silver nitrate solution, and then identified by preparing hexamethylenetetramine. His study greatly arouses our interest, especially from the biochemical point of view; he did not, however, investigate the form of formaldehyde in "Sotetsu."

(1) K. Yoshimura, *Bull. Kagoshima Agr. College*, **5** (1922), 25.

The writer[2] at first confirmed Yoshimura's experimental results by a different method, Rimini's and Vitali's colour reactions, and by preparing formaldomedon for identification. And then the writer found that the formaldehyde in "Sotetsu" is in the combined state of a new glucoside, and liberated it from the glucoside by the action of the emulsin in "Sotetsu."

So the writer intended to study the new glucoside containing formaldehyde and the sotetsu-emulsin. In the present paper, mainly some properties of sotetsu-emulsin are described.

I. Identification of Formaldehyde in Sotetsu-seed. Twenty hulled sotetsu-seeds were mashed and extracted with boiling 90% alcohol, and the alcoholic extract was evaporated to a small volume under reduced pressure. The distillate of the steam distillation of the above solution gave intensive Rimini's and Vitali's reactions, and 0.8 g. of its domedon crystallized out on adding 1.0 g. dimedon. The domedon thus obtained formed lustrous colourless prisms and melted at 188°C. (uncorr.), agreeing with formaldomedon.

II. The Form of Formaldehyde in Sotetsu-seed. Experiment A. A hulled sotetsu-seed was mashed and put into the thermostat at 35°C., and after 4 hours it was steam-distilled. The distillate gave intensive colour reactions for formaldehyde. Sulphuric acid was added to the residue of steam distillation and the distillation was continued further; in the second distillate, the formaldehyde reaction was inferior. **Experiment B.** A carefully hulled sotetsu-seed was boiled for 30 minutes under a reflux condenser, and treated in the same way as above. In this case, even in the first distillate the reactions of formaldehyde were very weak.

The results of quantitative estimation of formaldehyde are shown in Table 1.

Table 1.

Experiment	Sample (g.)	HCHO mg. in 1st distillate	HCHO mg. dissolved in boiling water
A	9.3374	13.3	—
B	8.4887	1.3	5.3

Thus, the formaldehyde in sotetsu-seed is an enzymatic decomposition product from its compound.

(2) K. Nishida, *J. Agr. Chem. Soc. Japan*, **11** (1935), 357.

III. Preparation of Sotetsu-emulsin. The hulled sotetsu-seeds were mashed and mixed thoroughly with an equal weight of water containing a small amount of toluene, and put into an ice-box. After one night, the mass was pressed on a cloth, and the filtered liquid was acidified with acetic acid to precipitate proteins. From the filtrate, the crude enzyme was precipitated with alcohol or acetone. The precipitate was washed with absolute alcohol or acetone, then with ether and finally it was dried in vacuo over sulphuric acid. For the further purification the resulting precipitate was redissolved in water and reprecipitated with alcohol or acetone and treated similarly to the above operation. By using acetone as precipitant, the yield of the emulsin-preparation from 4 kg. sample was 6.0 g.

IV. Usual Methods of Estimation of Enzyme Activity. The digestion mixtures usually consisted of 15 c.c. of 1% salicin solution, 5 c.c. of 0.4–0.8% sotetsu-emulsin solution, and 2 c.c. of Sörensen's buffer solution. The mixtures, preserved with toluene, were kept at 37°, usually for digestion hours of 90 minutes. Enzyme solution, inactivated by heat, were employed as controls in usual way. The enzyme activity has been measured by determining the reducing action of the decomposed salicin, e.g. with respect to Fehling's solution according to Bertrand.

V. Optimum *p*H of the Sotetsu-emulsin. In determining the *p*H optimum, citrate-HCl or phosphate buffer was used. The activity of

Table 2.

Salicin Hydrolysis at Various Hydrogen Ion Concentrations.

(15 c.c. of 1% salicin, 5 c.c. of 0.4% enzyme, 37°, 90 mm.)

*p*H	Standard $KMnO_4$ (c.c.)	Salicin hydrolysed (%)	$\frac{10^3}{t}\ln\frac{a}{a-x}$	$\frac{\varphi}{\Phi}$
3.6	3.10	16.8	2.04	0.52
4.3	5.05	27.7	3.60	0.92
4.5	5.15	28.3	3.70	0.95
4.8	5.35	29.5	3.88	0.99
5.1	5.40	29.7	3.91	1.00
5.2	5.20	28.6	3.74	0.96
5.8	4.90	26.9	3.48	0.89
5.9	4.80	26.3	3.39	0.87
6.1	4.55	24.8	3.17	0.81
7.0	2.45	13.2	1.57	0.40

sotetsu-emulsin at various values of *p*H are summarized in Table 2, in which the *p*H value indicates the mean of the initial and final reactions.

Thus the sotetsu-emulsin exhibited optimum activity for salicin hydrolysis, in the solution of *p*H 5.1. E. Vulquin[3] stated that almond-emulsin had an optimum activity at *p*H 4.4; whereas *p*H 4.7 to 5.1 for β-methylglucoside, 6.0 for amygdalin, and 4.2 for lactose were optimum for almond-emulsin according to Willstätter and Csányi.[4]

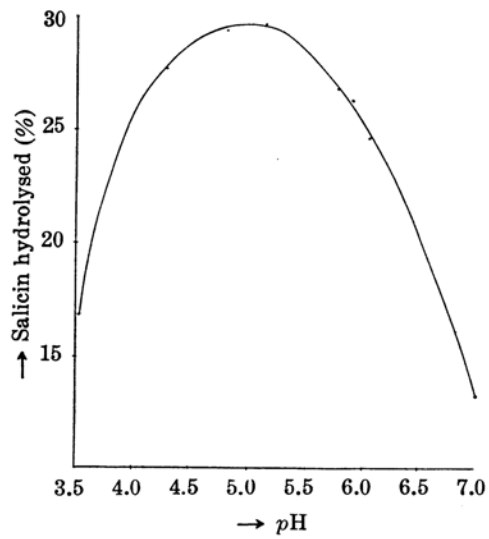

Fig. 1.

VI. Optimum Temperature of the Sotetsu-emulsin. The optimum temperature of sotetsu-emulsin was sought by using the usual mixtures of *p*H 4.8. Each of these mixtures was immersed in a water bath at different temperatures for 90 minutes. The rate of decomposition of salicin by sotetsu-emulsin at various temperatures was estimated as shown in Table 3.

Table 3.

Optimum Temperature for Salicin Hydrolysis by Sotetsu-emulsin.

(15 c.c. of 1% salicin, 5 c.c. of 0.8% enzyme, *p*H 4.8, 90 min.)

Temperature (°C)	Standard $KMnO_4$ (c.c.)	Salicin hydrolysed (%)	$\frac{10^3}{t}\ln\frac{a}{a-x}$	$\frac{\varphi}{\Phi}$
30	8.95	50.8	7.88	0.44
35	9.70	55.4	8.97	0.50
40	10.35	58.9	9.88	0.55
45	11.15	64.5	11.50	0.64
50	12.85	75.3	15.53	0.87
55	13.55	80.0	17.88	1.00
60	13.45	79.3	17.40	0.97
65	10.15	58.2	9.69	0.54

(3) E. Vulquin, *Soc. biol., Paris*, **70** (1911), 270, 763.
(4) R. Willstätter and W. Csányi, *Z. physiol. Chem.*, **117** (1921), 172.

According to the above results, the reaction velocity of the sotetsu-emulsin is accelerated as the temperature is raised to 55°C. On further elevation of the temperature the reaction velocity begins to diminish, and when the *p*H value of the solution is 4.8, we see that the sotetsu-emulsin may act at a maximum activity at 55–60°C.

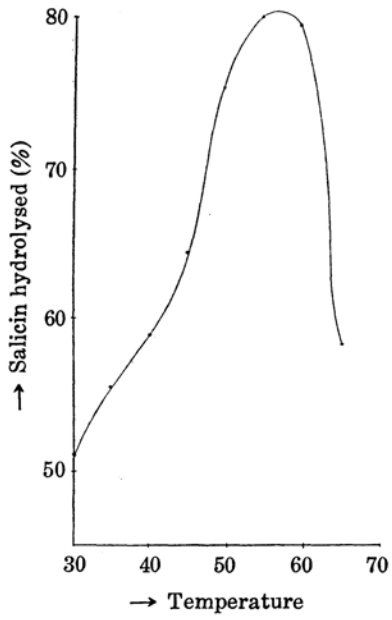

Fig. 2.

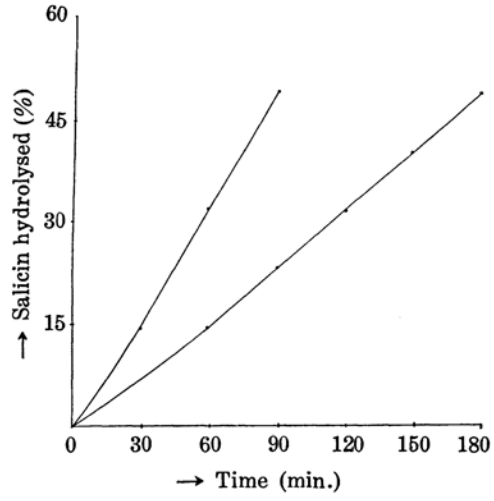

Fig. 3.

VII. Reaction Course of Sotetsu-emulsin. Hudson and Paine[5] studying the hydrolysis of salicin with almond-emulsin found that the reaction course of the enzyme follows the monomolecular reaction. J. Giaga[6] states that the reaction course of emulsin varies with the enzymes used and follows a sort of selective mass action law. Willstätter and Csányi[7] reported that the reaction of almond-emulsin for amygdalin hydrolysis was not found as a monomolecular reaction. And also according to Helferich's experiment,[8] in the reaction of Kahlbaum's emulsin-preparation for salicin hydrolysis, the monomolecular reaction constant increased in course of time. In our experiments with sotetsu-emulsin, as shown in table 4, we found almost the same tendency.

(5) Hudson and Paine, *J. Am. Chem. Soc.*, **31** (1909), 1242.
(6) J. Giaga, *J. chim. phys.*, **19** (1921), 77; *Chem. Abst.*, **16** (1922), 2336.
(7) *Loc. cit.*
(8) B. Helferich, *Z. physiol. Chem.*, **117** (1921), 159.

Table 4.

Kinetics of Salicin Hydrolysis by Sotetsu-emulsin.

(15 c.c. of 1% salicin, 5 c.c. of 0.4% enzyme, pH = 4.4, 37°)

Time (min.)	Standard $KMnO_4$ (c.c.)	Salicin hydrolysed (%)	$\frac{10^3}{t}\ln\frac{a}{a-x}$
60	2.70	14.2	2.55
90	4.30	22.9	2.89
120	5.80	31.4	3.14
150	7.25	39.7	3.37
180	8.80	48.9	3.73

VIII. Reaction Velocity and Enzyme Amount. According to Auld,[9] the reaction velocity of amygdalin hydrolysis by almond-emulsin is proportional to the concentration of the enzyme. Willstätter and Csányi[10] reported that the reaction velociy of hydrolysis of amygdalin, β-methyl-glucoside lactose and raffinose by enzyme is proportional to the amount of the enzyme. In our experiments, also on the decomposition of salicin the same relation was observed. The results are summarized in Table 5.

Table 5.

Reaction Velocity and Enzyme Amount. (pH = 4.4, 37°)

Substrate	Sotetsu-emulsin	Time (min.)	Standard $KMnO_4$ (c.c.)	Salicin hydrolysed (%)
15 c.c. of 1% Salicin	5 c.c. of 0.4% solution	60	2.70	14.2
,,	5 c.c. of 0.8% solution	30	2.70	14.2
,,	5 c.c. of 0.4% solution	120	5.80	31.3
,,	5 c.c. of 0.8% solution	60	5.85	31.7
,,	5 c.c. of 0.4% solution	180	8.80	48.9
,,	5 c.c. of 0.8% solution	90	8.80	48.9

IX. Total Hydrolysis of Salicin by Sotetsu-emulsin. Bertrand and Compton[11] reported that the salicin in concentration very much superior to that found in plants was completely hydrolysed by the action of emulsin. Also we found that glucose was completely splitted off from salicin, as shown in Table 6.

(9) S. J. M. Auld, *J. Chem. Soc.*, **93** (1908), 1251.

(10) *Loc. cit.*

(11) G. Bertrand and A. Compton, *Ann. Inst. Pasteur*, **39** (1925), 355; *Chem. Abst.*, **19** (1925), 2962.

Table 6.

Total Hydrolysis of Salicin by Sotetsu-emulsin.

(15 c.c. of 1% salicin, 5 c.c. of 0.8% emulsin, pH = 4.8, 37°)

Time (hour)	Standard $KMnO_4$ (c.c.)	Salicin hydrolysed (%)	$\frac{10^3}{t}\ln\frac{a}{a-x}$
5	15.70	94.5	9.67
7	16.15	97.5	8.79
8	16.50	100.0	9.59
15	16.50	100.0	—

Summary.

The principal experimental results described above are summarized as follows:

(1) Sotetsu-seed contains a glucoside of formaldehyde and this substance splits off formaldehyde by the action of sotetsu-emulsin.

(2) The emulsin was easily prepared from sotetsu-seed and some of its properties were studied in the case of salicin as substrate.

(3) The sotetsu-emulsin exhibited maximum activity in the solution of pH near 5.0.

(4) It was observed that the optimum temperature of sotetsu-emulsin for salicin hydrolysis is 55–60°C. near the optimum hydrogen ion concentration.

(5) In the reaction course of sotetsu-emulsin for salicin hydrolysis, the monomolecular reaction constant increased in course of time.

(6) The reaction velocity of salicin hydrolysis is proportional to the amount of sotetsu-emulsin.

(7) The salicin in 15 c.c. of 1% solution was completely hydrolysed by the action of sotetsu-emulsin in 5 c.c. of 0.8% solution, at the end of 8 hours in the digestion mixture of pH value 4.8 and temperature 37°C.

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