STUDY OF KINETICS OF ENZYMATIC HYDROLYSIS OF AMMONIUM GLYCYRRHIZATE

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Hydrolysis of glycyrrhizic acid (I) and its salts makes it possible to obtain glycyrrhetinic acid (II), having anti-inflammatory activity [3]. Up to the present time, the conditions of the hydrolysis have not been developed in detail. A prolonged residence of II in a mineral acid medium leads to the isomerization of β-II into the pharmacologically inactive α-isomer of II, and other derivatives [5]. Compound II can be preserved and isolated, using milder conditions, for example, enzymatic hydrolysis [8].

The study of the kinetics of the enzymatic hydrolysis of ammonium glycyrrhizate (III) by the action of β-glucuronidase (IV) was carried out on the "Ferment-1" apparatus [1, 2]. In the experimental work, we used a technical grade salt III as the substrate, which was produced according to the regulations for the production of the glycyrrham preparation, containing 97.2% of II. Grade B enzymatic preparation of IV, produced at the Olainsk factory of chemical reagents, with an activity of 58500 ED, TU 10P 408-70 was used. Acetate buffer solutions with pH values from 4.4 to 5.2 were used as the medium.

EXPERIMENTAL

To select the optimal conditions for the enzymatic hydrolysis, we carried out a calibration of the apparatus with reference to II. For this, a series of experiments were carried out, recording changes in the electrical conductivity, corresponding to the chemical conversion of III by the action of IV. A 155 ml portion of a 0.003 M solution of III in an acetate buffer solution at pH 4.6 was prepared. Portions of 2.5 ml of this solution were poured into a pair of cuvettes for measuring the electrical conductivity, and the cuvettes were placed into housings in the thermostat at 40°C. The remaining 150 ml of the substrate were placed at 40°C into the thermostat. The enzymatic reaction was started simultaneously in the cuvette, by adding 0.2 ml of 1% solution of the enzyme into the experimental cuvette and 0.2 ml of the acetate buffer into the control cuvette, and in the flask, whereto 12 ml of a 1% solution of IV were added. Every 10 min (in the course of 60 min), 25 ml aliquots were withdrawn and heated to 80-85°C to stop the enzymatic reaction. After cooling the solution was acidified by 0.1 N hydrochloric acid. The precipitate of II was separated and washed on the filter with hot distilled water to remove unreacted III. The precipitates obtained on the filter were dried, and then dissolved in 10-15 ml of 95% alcohol. The solutions were filtered into 100 ml volumetric flasks and brought to the mark with ethanol; a 50 ml portion of the solution obtained was transferred into a 100 ml volumetric flask and again brought to the mark with 95% ethanol. A quantitative spectrophotometric determination was carried out at the wavelength of 248 nm.

After 60 min, the sensors were disconnected, and on the self-recorder tape of the millivoltmeter the value of the deviation was measured (in millimeters) at the same periods of time at which the aliquots were withdrawn.

TABLE 1. Determination of Calibration Coefficient N during Enzymatic Hydrolysis of Ammonium Glycyrrhizate III

Time of thermostating, min	Optical density of solutions at 280 nm	Glycyrrhetinic acid content, mg	Deviation of self-recorder, mm	$\frac{M}{P}$	N_{mean}
10	0,280	2,6046	18	0,1447	
20	0,520	4,7878	31	0,1544	
30	0,820	7,5262	49	0,1535	
40	0,870	7,9976	50	0,1599	0,153
50	0,950	8,7866	57	0,1541	
60	1,030	9,6256	62	0,1553	

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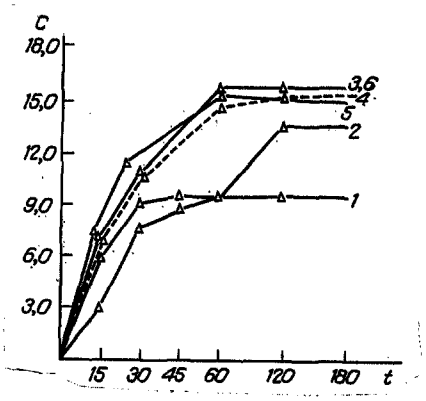

Fig. 1

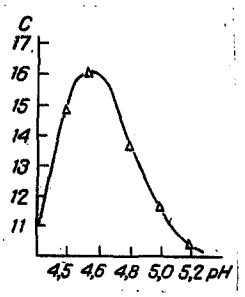

Fig. 2

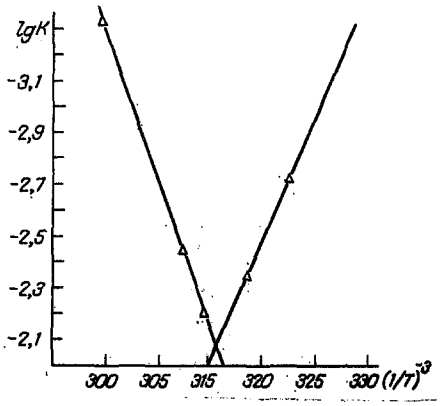

Fig. 3

Fig. 1. Graph for relationship between the amount of the enzymatic preparation introduced and the yield of II. On abscissa, time of thermostating (in min); on ordinate, amount of II separating out in the hydrolysis of a 0.003 M solution of III (in mg). Amount of 1% enzyme preparation of IV introduced: 1) 0.1 ml; 2) 0.2 ml; 3) 0.3 ml; 4) 0.4 ml; 5) 0.5 ml; 6) 0.6 ml.

Fig. 2. Graph for the dependence of activity of enzymatic preparation of IV on pH. On abscissa, pH; on orginate, amount of II separating out (in mg).

Fig. 3. Dependence of logarithm of rate constant of hydrolysis of III by β-glucuronidase (IV) on 1/T.

TABLE 2. Yields of Glycyrrhetinic Acid II after Enzymatic Hydrolysis of Ammonium Glycyrrhizate III

Number of flask	Weight of precipitate after hydrolysis, g	Yield of purified II	
		g	%
1	0,0726	0,0634	47,49
2	0,0731	0,0642	48,08
3	0,0725	0,0638	47,47
4	0,0720	0,0630	47,19
5	0,0718	0,0640	47,94
6	0,0012	—	—

From the amount of II which separated out (M) and the distance by which the self-recorder pen had deviated (P) during the same period of time, the amount of II was determined which corresponds to a 1-mm deviation of the self-recorder pen from the formula $N = \frac{M}{P}$. The data are listed in Table 1. It is seen that a 1-mm deviation of the self-recorder pen corresponds to 0.153 mg of II.

The above calibration of the apparatus made it possible subsequently during the selection of the optimal hydrolysis conditions to express the yield of II in accordance with the graphical recordings obtained.

Determination of Optimal Concentrations of Substrate and Enzyme. The enzymatic reactions are usually carried out by using a concentration of the substrate in the range 10^{-2}–10^{-3} M. We used 0.003, 0.006 and 0.012 M solutions of III. The analysis of the experimetal data obtained showed that with increase in the substrate concentration, the yield of the hydrolysis products decreases at all pH values from 4.5 to 5.2. This is explained by the fact that the solutions of III have a viscous consistency, and therefore at concentrations of 0.006 and 0.012 M, the rate of the enzymatic reaction decreases. The optimal results were achieved during the hydrolysis of 0.003 M solutions of III, and further decrease in the concentration of the substrate did not lead to an increase in the rate of hydrolysis.

To find the optimal amount of the enzyme preparation, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 ml of a 1% solution of IV were added into the experimental cuvettes each containing 2.5 ml portions of the substrate at different concentrations. Figure 1 shows graphically the relation between the amount of the enzymatic preparation introduced and the compound II separating out. It is seen that the optimal concentration of the enzyme in the reaction solution is reached when 0.3 ml and more of a 1% solution of IV are added to a 0.003 M solution of III.

During the hydrolysis of 2.5 ml of a 0.012 M solution of III by a small amount of the enzymatic preparation (0.05-0.2 ml), the yield of the hydrolysis products is 2-3%; increase in the amount of the enzyme introduced to the reaction up to 0.6 ml increases the yield of the hydrolysis products threefold.

Determination of the Optimal Value of pH of the Medium. The optimal value of pH for slightly purified preparations of IV obtained from animal tissues varies from 4.5 to 5.2 [4, 6, 7]. We carried out a hydrolysis of a 0.003 M solution of III by a 1% solution of the enzymatic preparation of IV in an acetate buffer at different pH values from 4.5 to 5.2 from the data obtained a graph was plotted, shown in Fig. 2, which shows that the optimal pH value for the preparation IV is 4.6; at this pH value the maximum yield of II was obtained.

Determination of Optimal Temperature of Enzymatic Process. Portions of 2.5 ml of a 0.003 M solution of III were placed in cuvettes. Into the experimental cuvette, 0.3 ml of a 1% solution of IV was introduced and into the control cuvette 0.3 ml of the acetate buffer. The thermostating was carried out for 120 min at 30, 37, 40, 45, 50, and 60°C.

The hydrolysis rate constants were determined at different temperatures and were calculated according to a first-order reaction equation.

$$K = \frac{-2.303}{t} \cdot \lg \frac{C}{C_0},$$

where C_0 is the initial concentration of III; C is the amount of III which did not undergo a hydrolysis during time t; t is time of carrying out the hydrolysis.

A graphical dependence of log K on 1/T is shown in Fig. 3. The graph has the form of a line with inflection. This feature of the influence of the temperature on the reaction kinetics is explained by the accleration of the denaturation of the protein molecule, leading to inactivation of the enzyme at elevated temperatures. In the 37-50°C range the yield of II is the highest.

With the data on the kinetics of the enzymatic hydrolysis which we obtained, it was possible to develop a method of hydrolysis of I and isolation of II. Portions of 100 ml of 0.003 M solution of III in an acetate buffer at 4.6 were placed in 6 flasks, and the flasks were held for 2-3 h at 40°C. Then 12 ml portions of 1% enzyme solutions were introduced into 5 flasks, while 12 ml of an acetate buffer (control) were added to the 6th flask, and the flasks were thermostated for 2 h. At the end of the reaction, the solutions were heated to 80-85°C to inactivate the enzyme, and were acidified with a 0.1 N solution of hydrochloric acid to pH 3.0-3.5. The precipitate was separated, washed with hot distilled water, and then dried, and the pure II was crystallized from an alcohol–water (1:1.5) mixture. Data on the yield of the purified II after drying at 80-85°C are given in Table 2.

The enzymatic hydrolysis carried out in an optimal regime makes it thus possible to obtain II in a yield of 48%.

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