CONTINUOUS HYDROLYSIS OF SOLUBLE STARCH BY IMMOBILIZED AMYLOGLUCOSIDASE (E.C. 3.2.1.3)

J. KUČERA

Research Institute of Food Industry, 150 38 Prague 5

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The hydrolysis was examined of starch by immobilized amyloglucosidase (EC 3.2.1.3) in a continual stirred tank reactor at 60°C. The experimental data were treated according to different approximative kinetic relations. As follows from the results of calculations of total enzyme activity in the reactor and of the enzyme activity determined by conventional analytical methods, all the approximative procedures lead practically to identical results. The effects were determined of long-term operation of the continual stirred tank reactor on the activity of the immobilized enzyme and the kinetic constant of thermal enzyme inactivation during the operation of the reactor was evaluated ($k_d = 2{\cdot}211 \text{ min}^{-5}$). The plot of the rate of hydrolysis of maltooligosaccharides by immobilized amyloglucosidase *versus* the polymerization degree of the oligosaccharide shows a profile which is different from the profile of the plot characterizing the rate of hydrolysis of the same oligosaccharides by soluble amyloglucosidase.

The hydrolysis of starch to glucose catalyzed by amyloglucosidase is one of the reactions routinely effected by immobilized enzymes[1-13]. Most of the conventional supports, such as agarose, cellulose and its derivatives, polyacrylamide, glass *etc.* have been used in this reaction. The coupling of amyloglucosidase to these supports has also been effected by practically all the immobilization methods. The course of the reaction examined for long-term periods of continual process[5,6], however, has been reported in a few cases only.

This study was designed to examine the kinetics of hydrolysis of soluble starch to glucose, catalyzed by amylglucosidase immobilized by covalent coupling to a carboxymethyl-cellulose gel[14], in a continual stirred tank reactor (CSTR), to investigate the inactivation of the enzyme during a long-term operation, and to study the effect of the polymerization degree of maltooligosaccharides on the reaction rate. The fundamentals of the kinetics of enzyme reactors and of inactivation of immobilized enzymes in continual processes have been reported in a number of original papers and summarized recently in a review article[15]. The results were therefore treated according to well-known relations discussed elsewhere.

The data presented here show the use of an enzyme reactor of the CSTR type for the preparation of glucose from starch and may serve also as a model design of this reactor type for hydrolyses by immobilized enzymes.

EXPERIMENTAL

Carboxymethyl-cellulose gel was prepared as described earlier[14] from Lovosa KMC TS 05 carboxymethyl-cellulose (Severočeské chemické závody, Lovosice). This gel contains approximately 2 mmol of carboxymethyl groups per 1 g of dry weight and the dry weight of the hydrated gel is c. 8%). The gel structure is suitable for an effective coupling of amyloglucosidase (mol. wt. c. 45000) and permits the substrate to diffuse through the gel at a high rate. This can be documented by a relatively high effectiveness factor (0·82).

Amyloglucosidase used in this study was from Amayo Pharm. Co., Kyoto, Japan, and its specific activity was 26 EU/mg. This high specific activity permits the coupling of the enzyme to be performed without any preliminary purification.

The hydrolysate of starch used in this study was prepared from technical potato starch by treatment with bacterial α-amylase. The mean polymerization degree of the hydrolysate was c. 12, the mean molecular weight 1960. The concentration was 30%.

The concentration of glucose was determined by a combined method using glucose oxidase and peroxidase[14].

The enzyme reactor was adapted from a fermentor, supplied as part of the apparatus for continual cultivation of microorganisms (Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague). A schematical representation of the reactor is given in Fig. 1. The reactor was provided with a glass filter (G 1, diameter 12 mm), placed close to the stirrer. The reactor was closed up and the reaction mixture was pumped by a sampling pump to the product reservoir through tubing leading from the filter. The constant temperature of the reactor was controlled by a Höppler ultrathermostat. The flow rate was checked at irregular intervals by measuring the volume of liquid which passed through the reactor in 1 min. The reactor, whose total volume was 3000 ml, was filled with 1000 ml of substrate.

Amyloglucosidase activity of the immobilized enzyme was determined by mixing 1 ml of the immobilized enzyme with 9 ml of 4% solution of soluble starch. The suspension was placed on a shaker for 30 min at the temperature chosen (30 or 60°C). The enzyme was filtered off afterwards and the quantity of glucose formed was determined.

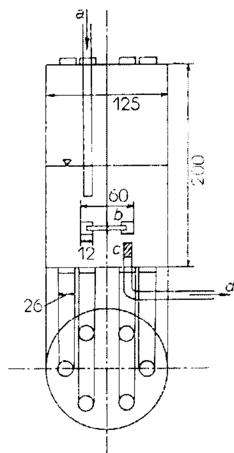


FIG. 1

Reactor 1000 ml in Volume

a Substrate inlet, *b* stirrer, *c* filter, *d* product outlet.

RESULTS AND DISCUSSION

Having regard to the results of the orienting experiments the reaction was allowed to proceed at 60°C and a retention time of 30 min. The concentration of substrate used expressed in molarity of bonds hydrolyzable was 0·15M (according to end group analysis). The total activity of the immobilized enzyme in the reactor can be calculated from this concentration and retention time K'_m by any of the procedures described[15]. The determination of K_m has been reported earlier[14]. The concentrations of glucose determined in the reaction mixture are given in Table I. The values were examined during 24 h of continual operation at 60°C and various retention times. In subsequent calculations an arithmetic mean was considered of the values measured at a retention time of 30 min, marked by a mean relative error of 5·83% (8 measurements).

The calculation was carried out first according to the simplest formula derived by O'Neille[6,16] for a reaction of zero order

$$q(S_0 - S_K) = k_2E\,, \tag{1}$$

where q is the flow rate of the substrate solution through the reactor (ml/min), S_0 the initial substrate concentration, S_K the final substrate concentration, and k_2E the total enzyme activity in the reactor. Inserting the values from Table I into this relation we arrive at 53 579 EU as total enzyme activity in the reactor. Since 100 ml of immobilized enzyme were used in the reactor, its activity is 536 EU/ml according to this determination. When the activity is determined as described under Experimental, a value of 59 EU/ml is obtained for 30°C and a value of 545 EU/ml for 60°C. The value of 536 EU/ml obtained by a calculation based on data for the continual reactor is in good agreement within experimental error (5·83%) with the analytical activity determination at 80°C. The substrate for the analytical determination was not identical with the substrate used in the reactor and showed a slightly higher mean polymerization degree ($\overline{DP}$ = 18). The differences in the rates of hydrolysis of the

TABLE I

Hydrolysis of Soluble Starch in Continual Stirred Tank Reactor 1 000 in Volume
Initial substrate concentration (S_0) = 1 650 mM, temperature 60°C.

Retention time, min	30	60	90	120
Final substrate concentration, mM	41	10	5	0
Final product concentration, mM	1 609	1 640	1 645	1 650
Conversion, %	97·52	99·39	99·69	100·00
Mean relative error, %	5·83	6·02	5·94	6·15

substrate corresponding to this difference in polymerization degree do not play, however, a role because of the short time period during which activity is determined and because of the considerable excess of substrate when the reaction is carried out on analytical scale. The degree of conversion does not exceed the order of units of per cent and for this reason too the influence of the polymerization degree on reaction rate is negligible.

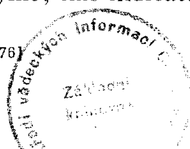
The total enzyme activity in the reactor can be calculated also with the aid of a more complicated relation[17,18] involving the effect of the apparent Michaelis constant (K'_m). The agreement of the results obtained by using equation (*1*) and (*2*) indicates that the value of the Michaelis constant does not play a role in this reaction which thus follows the kinetics of zero order.

$$(S_0 - S_K)(K'_m + S_K)/S_K = k_2 E/q \,. \tag{2}$$

Introducing into equation (*2*) the values from Table I we arrive at 53 827 EU as total enzyme activity in the reactor and at 538 EU/ml as activity of the immobilized enzyme, *i.e.* at values which are in good agreement with both the results of the analytical activity determination and also with results obtained by using equation (*1*).

Since the analytical determination of the activity of the immobilized enzyme was carried out with a volume of 10 ml and its results are in good agreement with the results of continual operation of a reactor 1000 ml in volume, this agreement can be regarded as surprisingly good. At the same time this finding points to the possibility of increasing the reactor volume up to hundred times by mere calculation, at least in cases where the reaction follows zero order kinetics as was the case in this study.

The high substrate concentrations used for these experiments practically eliminate diffusion effects. To judge the role of diffusion the inlet substrate concentration was decreased in subsequent experiments to a value approximating the order of K'_m (1·65 mM and 3·3 mM). This extremely low substrate concentration required that the quantity of immobilized amyloglucosidase be likewise decreased. Since the quantity of the immobilized enzyme in the reactor could not be decreased adequately, an enzyme of lower specific activity, immobilized by the method described earlier[14], was used. This procedure yielded amyloglucosidase of an activity 6 EU/ml at 60°C (the activity was lower than 1 EU/ml at 30°C). The volume of this immobilized enzyme of low activity added to the 1000-ml reactor was 5 ml, *i.e.* the total activity of the enzyme in the reactor was 30 EU/ml at 60°C. The K'_m-value of the immobilized enzyme, the K_m-value of soluble amyloglucosidase (0·454 mM), and the E_{ov}-value (0·82) have been reported earlier[14]. The R_{ov}-value, which can be affected by the type of bond, was checked experimentally. The remaining values were taken from the original communication[14]. The conversion is extremely high even under these conditions (Table II). The Michaelis constant of immobilized amyloglucosidase is lower than the same constant of the soluble enzyme; this indicates the effect of the distri-



bution coefficient (K) of the substrate characterizing its distribution between the liquid phase and the support. This coefficient can be evaluated by the relation derived by Rony[18]

$$K'_m/K_m = (KE_{ov})^{-1} . \quad (3)$$

The value computed from this relation is $K = 2{\cdot}93$, *i.e.* the substrate concentration of the support is considerably higher than the substrate concentration of the liquid phase at equilibrium state. The total enzyme activity in the reactor can therefore be calculated by using the K_m of the native soluble enzyme together with the distribution coefficient and the effectiveness factor (E_{ov}). Rony[18] derived the following relation for low substrate concentration where diffusion effects play a role

$$S_o/S_K = 1 + (KE_{ov}Ek_2/K_m q) . \quad (4)$$

Inserting the experimental results (Table II) into this equation we obtain a value of 28·69 EU (*i.e.* c. 29 EU for total enzyme activity in the reactor; since 5 ml of the immobilized enzyme was used, we arrive at an activity of 5·74 EU/ml, a value which is in agreement with the analytical result (6 EU/ml).

Kobayashi and Moo-Young[19] derived the following equation for low substrate concentrations and a continual stirred tank reactor (CSTR)

$$1 - S_K/S_0 - k_L a\tau/2\varepsilon^2[S_K/S_0 + k_2E(1 - \varepsilon)/k_L aS_0 + K_m/KS_0 -$$
$$- \{(S_K/S_0 - k_2E(1 - \varepsilon)/k_L aS_9 - K_m/KS_0)^2 - 4K_m S_K/KS_0^2\}] , \quad (5)$$

where τ is the retention time and ε the free reactor volume relative to the total volume. Using the data given in Table II for retention time 30 min and substrate concentration 3·3 mM we obtain the value of total rate constant of mass transfer ($k_L a$). Its value is $k_L a = 61{\cdot}53 \text{ cm}^{-2} \text{ min}^{-1}$.

It has often been reported in literature that the stability of immobilized enzymes is higher than the stability of native soluble enzymes. Rarely, however, is the stability of the immobilized enzyme characterized by the rate constant of thermal denaturation and often is the stability measured in a short-term experiment only. The results of a continual, long-term experiment are more reliable, however, and eliminate certain complicating phenomena, such as, *e.g.* the increase of diffusion rate brought about by higher temperature, an increase which can partly compensate for short periods the decrease of reaction rate caused by enzyme inactivation. We have carried out long-term, continual experiments in the stirred tank reactor at 3·3 mM substrate concentration and initial total activity of immobilized amylglucosidase 30 EU. The experiments were continued for 18 days. The concentration of the product in the reaction mixture was checked at regular intervals.

The results summarized in Table III were used to compute the rate constant of thermal denaturation of the enzyme (k_d) according to the fundamental relation derived by O'Neille[17] and characterizing the kinetics of thermal denaturation of immobilized enzyme in a continual stirred tank reactor:

$$\ln (S_{Kt}/(S_9 - S_{Kt})) - \ln (S_K/(S_0 - S_K)) = k_d t . \quad (6)$$

TABLE II

Hydrolysis of Soluble Starch in Continual Stirred Tank Reactor 1 000 ml in Volume

Initial substrate concentration S_0 = 1·65 and 3·3 mM, temperature 60°C.

Retention time, min	30		60		90	
S_K, mM	0·298	0·602	0·165	0·329	0·090	0·182
S_0, mM	1·650	3·300	1·650	3·300	1·650	3·300
P_K, mM	1·352	2·698	1·485	2·971	1·560	3·118
Conversion, %	81·93	81·76	90·00	90·03	94·54	94·48
Mean relative error, %	5·80	5·64	5·92	6·14	6·02	5·64

TABLE III

Thermal Denaturation of Immobilized Amyloglucosidase in Continual Stirred Tank Reactor

Time days	P_K mM	S_K mM	Conversion %
0	2·698	0·602	81·76
1	2·675	0·625	80·06
2	2·659	0·641	80·58
3	2·642	0·658	80·06
4	2·625	0·675	79·55
7	2·574	0·726	78·00
8	2·556	0·744	77·45
9	2·538	0·762	76·91
10	2·521	0·779	76·39
11	2·502	0·798	75·82
14	2·444	0·856	74·06
15	2·423	0·877	73·42
16	2·403	0·897	72·82
17	2·383	0·917	72·21
18	2·361	0·939	71·55

This relation can be linearized (the plot of ln $S_{Kt}/(S_0 - S_{Kt})$ *versus* time is linear with k_d as constant) and the results were therefore evaluated by the method of least squares. Symbol S_{Kt} designates final substrate concentration after time t. The treatment of the data by the method of least squares yielded $k_d = 2{\cdot}211 \,.\, 10^{-5}\,\text{min}^{-1}$. This procedure, however, cannot be applied to the soluble enzyme and therefore the stability of soluble and immobilized amyloglucosidase cannot be quantitatively compared. Nevertheless it can be said almost for sure that the native enzyme would have been completely inactivated after 18 days at 60°C.

The kinetics of relations catalyzed by immobilized enzymes is afffected by the diffusion of the substrate through the support. Since the substrate of amylglucosidase is an oligosaccharide, we may expect that a role will play the effect of mean polymerization degree on the diffusion rate and thus also on the rate of the enzymatic reaction. The dependence of the reaction of soluble amyloglucosidase on the polymerization degree of oligo-maltodextrin is sufficiently known. The rate does not depend on the polymerization degree if this degree is higher than 6. At lower polymerization degrees the reaction rate decreases with the decreasing polymerization degree. The reaction catalyzed by immobilized amyloglucosidase must necessarily follow the same course, yet it is complicated by the simultaneous effect of diffusion. By contrast, the diffusion rate increases with the decreasing molecular weight. These two actions proceeding simultaneously can cause that the behavior of the immobilized enzyme is different from the behavior of the native soluble enzyme. To judge these effects we prepared a substrate solution of mean polymerization degree 4·6, 5·2,

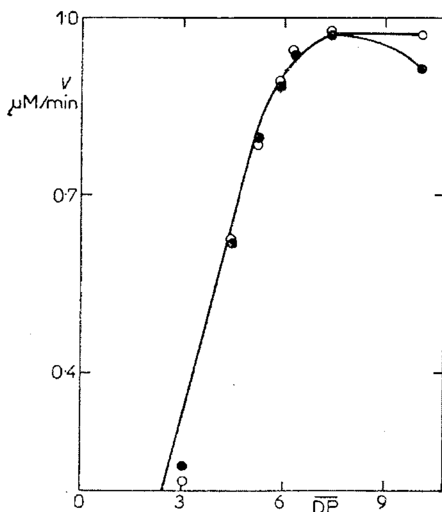


FIG. 2

Plot of Hydrolysis Rate of Starch Hydrolyzed to Different Degree *versus* Polymerization Degree

Full circles hydrolysis by immobilized amyloglucosidase; empty circles hydrolysis by soluble amyloglucosidase.

5·9, 6·4, 7·6, and 10·1. The dependence of reaction on polymerization degree was well observable over this range both with immobilized and also with native, soluble amyloglucosidase. Both plots are shown in Fig. 2. Whereas the reaction rate of the reaction catalyzed by soluble amyloglucosidase increases up to a mean polymerization degree of approximately 8 and does not change above this degree, the rate of the reaction catalyzed by immobilized amyloglucosidase increases in the same manner up to a polymerization degree of approximately 8 and then slightly decreases with the increasing polymerization degree. The differences in reaction rates, however, are very small and cannot be used for quantitative characterization of the effect of diffusion. In spite of that is the difference in reaction rates at a mean polymerization degree of 10·1 statistically significant according to the Student *t*-test (confidence degree 0·95). A quantitative characterization of the effect of polymerization would require most likely a substrate of higher polymerization degree. The substrate used was a hydrolysate, characterized by mean polymerization degree, and not an individual oligo-maltodextrin. The results obtained do not correspond therefore exactly to recorded data on the soluble enzyme. The relation between the soluble and immobilized enzyme, however, is not influenced by this fact.

The data obtained show that the approximative methods of calculation of kinetics of reactions catalyzed by immobilized enzymes yield practically identical results regardless of the method by which the equations have been derived. These methods can be used for increasing the volumes of the reactors without any other experimenting. The reproducibility of the results is excellent even if the volume is increased one hundred times. The stability of amyloglucosidase immobilized on carboxymethyl-cellulose gel is good. It has been shown that the polymerization degree of the starch hydrolysate used as a substrate of immobilized amyloglucosidase affects the rate of hydrolysis to glucose and that differences exist between immobilized and soluble amyloglucosidase as regards the dependence of reaction rate on the polymerization degree. These differences can be well accounted for by the effect of diffusion.

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