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THE THERMOSTABILITY OF GLUCOAMYLASE IMMOBILIZED IN DIFFERENT WAYS HAS A CERTAIN LIMIT*

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Summary

Thermostability of glucoamylase from *Aspergillus niger* is increased both by immobilization and substrate binding. However, the total superimposed stabilization effect at a given condition is apparently restricted by a certain limit which hardly depends on the mode of immobilization of the enzyme, and is determined mostly by the enzyme-substrate complex formation.

In the last decade, glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) has received considerable attention owing to its potential industrial usefulness in the immobilized form for the conversion of starch maltodextrines into glucose. Special attention is paid thereby to the production of immobilized glucoamylase that possesses a sufficiently high thermostability at high temperature (especially the temperature of pasteurization, i.e. 60—65°C). We believe that immobilized glucoamylase that has a half-life of about 3—4 weeks (or more) at 65°C could be considered a technologically feasible preparation. However, in spite of a great number of papers published on immobilization of this enzyme, the half-life of most stable insoluble preparations at 65°C do not exceed 5—7 days [1,2]. The question arises whether this value characterized a certain limit of thermostability of glucoamylase. This is not an idle question, because a verification of the answer, negative or positive, will stimulate the search for basically new approaches to production of immobilized glucoamylase with pregiven thermostability.

In the course of our studies [2—4] we concentrated on the effect of stabilization of glucoamylase against thermal action by the substrate (malto-

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dextrines). Glucoamylase derived from *Aspergillus niger*, manufactured by NOVO Industries A/S, Copenhagen, Denmark, was used in the experiments. The specific activity of the enzyme with respect to soluble starch was 9000 ± 500 μmol/min per g (150 ± 10 μkat per g) at 25°C. Starch hydrolysate of 24 D.E. called STAR-DRI 24-E, which is a partially acid hydrolyzed cornstarch (product of A.E. Staley Company, Decatur, IL) was used as maltodextrines. Controlled pore SiO_2 (average pore diameter 1150 Å) and porous glass (pore diameter 750 Å) as well as the enzyme preparation and the corn syrup solids were kindly given to us by Mr. H. Weetall of Corning Glass Works for which we are greatly indebted. Gossipol (dialdehyde, for the chemical structure see Table I) was kindly given by Dr. M.M. Rakhimov, Central Asia Research Institute for Food Industry, USSR.

Immobilization of glucoamylase on SiO_2 carrier by the silane-glutaraldehyde method was carried out as described in [1]. The inactivation kinetics of immobilized glucoamylase was mostly studied at 75°C in a thermostatted column (15 × 0.5 cm) packed with 60—100 mg of the immobilized enzyme. The experiment was devised in such a way that the degree of conversion of the substrate at the output of the column never exceeded 30%. Glucose in the reaction products was determined by means of the glucose oxidase-peroxidase method [5]. With the amount of immobilized glucoamylase ranging from 30 to 100 mg, a linear dependence of the substrate conversion on the weight of the enzyme preparation was observed. The experiment is described in detail in Refs. 3 and 4.

We have found that the major role in the stabilization of glucoamylase from *A. niger* belongs to the substrate binding (40-fold decrease of the first-order inactivation rate constant on the formation of the complex both with maltodextrines and maltose at 75°C) rather than immobilization of the enzyme on an inorganic support, for example, controlled pore silica or porous glass (3—6-fold decrease of the inactivation rate constant at 75°C). Moreover, substrate stabilization is expressed to a greater degree with respect to soluble glucoamylase (40-fold) than with respect to the immobilized enzyme (15-fold). It should be emphasized that the total limits of the thermostability increase (at the expense of immobilization and/or substrate stabilization) are close for the soluble and immobilized enzyme and is 50—100 times. As was shown, the substrate stabilizing effect does not depend on the length of the substrate carbohydrate chain, both for maltose that contains two glucose units, and STAR-DRI 24-E maltodextrines (A.E. Staley Company, Decatur, IL) that contain on an average seven glucose units per oligosaccharide molecule [4], stabilize glucoamylase to the same degree.

These data allows to conjecture that the thermostability of glucoamylase immobilized on inorganic supports has a certain limit which hardly ever depends on the mode of immobilization. This limit is mostly determined by the stabilization of the enzyme by cornstarch maltodextrines. The data that support this suggestion were obtained by us [2] when we studied the thermoinactivation of immobilized glucoamylase modified in 13 different ways. Apart from the enzyme immobilized on porous SiO_2 or porous glass by the silane-glutaraldehyde method we also obtained preparations in which the Schiff base formed was reduced with sodium borohydride, and then the

preparations where glucoamylase coupled to SiO_2 or porous glass by a silane-glutaraldehyde method was modified by either acroleine, succinic anhydride, glutaraldehyde, dimethyl adipimidate, carbodiimide/tetramethylenediamine, carbodiimide/hexamethylenediamine, carbodiimide/dodecamethylenediamine, carbodiimide/glucosamine, succinic anhydride/glucosamine, and by maltodextrines oxidized with HIO_4. However, in spite of the fact, that with some of the modifications we observed increase in the thermostability of immobilized preparation (3—7-fold), the substrate on being bound with glucoamylase induced some additional increase in the thermostability which resulted in an almost unchanged (or somewhat lower) half-life of the immobilized enzyme. In other words, in all the cases of immobilization of glucoamylase on inorganic supports studied by us (with the supports and the modes of chemical modifications of the immobilized enzyme being varied) the main contribution to the increase in thermostability was as a rule made by the enzyme-substrate binding. Thereby, depending on this "prearrangement" of the enzyme induced by an immobilization, the substrate stabilization was higher or lower in its absolute value but almost in all the cases a certain limit in the thermostability of glucoamylase was observed.

This conclusion seems to be very important both from the theoretical and technological point of view, for it allows to reveal the realistic possibilities of the increase in the resistance of glucoamylase to thermal action. In order to get some more evidence for it, we have studied the thermostability of this enzyme immobilized through various linkages (glutaraldehyde, acryloylchloride, and gossipol) and on different supports (SiO_2, polyacrylamide gel, and a combination of the two). The data obtained are summarized in Table I. It can be seen that the inactivation rate constants for the preparations are rather close and in any case no basically different effects in the stabilization of immobilized enzyme were revealed. The effect of additional stabilization of glucoamylase on immobilization in the presence of 30% maltodextrines which was first reported in [2], varies in the values for various batches of the immobilized enzyme and is not sufficiently reproducible.

It should be concluded from what has been discussed above that the thermostability of the known preparations of immobilized glucoamylase described in literature and ours is apparently restricted by a certain limit which is determined first and foremost by the substrate binding rather than by the mode of immobilization of the enzyme. As far as we know this is the only example in the practice of enzyme immobilization where a certain limit of thermostability of a fixed enzyme apparently has been revealed. The authors would be very glad if this major conclusion of the paper will not be substantiated by future studies of glucoamylase immobilization as, in this case, there will appear a real possibility for the economically expedient application of fixed glucoamylase for high-temperature conversion of starch maltodextrins into glucose on an industrial scale.

TABLE I

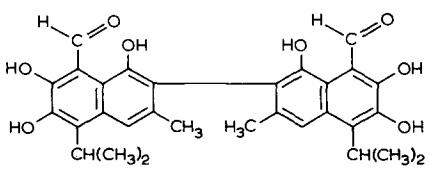
THERMOSTABILITY OF GLUCOAMYLASE FROM *ASPERGILLUS NIGER*, IMMOBILIZED ON INORGANIC AND ORGANIC SUPPORTS

Inactivation was carried out at 75° C, pH 4.5, in the presence of 30% cornstarch maltodextrines.

Carrier	Immobilization technique	$k_{in(app)}$ min^{-1} ($\times 10^3$)
—	Soluble glucoamylase (in the absence of the substrate)	1000 ± 100
—	Soluble glucoamylase	27 ± 3
SiO_2	Silane-glutaraldehyde	9.0 ± 0.5*
SiO_2	Silane-glutaraldehyde followed by acryloylchloride modification	9.9 ± 0.7
SiO_2	Silane-glutaraldehyde (in the presence of 30% maltodextrines)	5 ± 2
SiO_2	Silane-gossipol**	7.9 ± 0.6
SiO_2	Silane-gossipol (in the presence of 30% maltodextrines)	7.3 ± 0.7
Porous glass	Silane-glutaraldehyde	7.2 ± 0.5
Porous glass	Silane-glutaraldehyde followed by reduction with $NaBH_4$	6.4 ± 0.4
Porous glass	Silane-glutaraldehyde followed by glutaraldehyde cross-linking	7.9 ± 0.4
Porous glass	Silane-glutaraldehyde followed by carbodiimide/ glucosamine	7 ± 1
Porous glass	Silane-glutaraldehyde followed by coupling with maltodextrines oxidized with HIO_4	8.7 ± 0.5
20% PAAG***	Acrolein	13 ± 1
30% PAAG	Acrolein	11 ± 1
30% PAAG	Acryloylchloride	5.2 ± 0.4
30% PAAG	Acryloylchloride (in the presence of 30% maltodextrines)	5.2 ± 0.5
SiO_2 + 20% PAAG	Silane-glutaraldehyde followed by acryloylchloride modification and then by covalent coupling of the immobilized enzyme to PAAG	5.5 ± 0.5

*The constant can vary up to 20 $\cdot 10^{-3} \cdot min^{-1}$ for different batches of immobilized glucoamylase depending on the immobilization conditions.

**Dialdehyde



***Polyacrylamide gel.

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