Zu beachten ist, daß man den Überschuß an Diazomethan nicht größer wählt als angegeben, da sonst die dunkelbraunen, öligen Additionsprodukte von Diazomethan an Chinone, die stets auftreten, zum Hauptumsetzungsprodukt werden. Wichtig ist außerdem, daß man eine durch Destillation hergestellte Diazomethanlösung verwendet, da die übliche, durch Dekantieren des Äthers von der alkalischen Entwicklungslosung dargestellte Lösung stets noch so viel Alkali enthält, daß vorhandenes Chinon zersetzt wird.

Nach 12—15 Std. zieht man den Äther von der nun Methoxychinon enthaltenden Lösung ab; es verbleibt ein dunkelbrauner, fester, manchmal auch schmieriger Rückstand, das noch stark verunreinigte Methoxychinon.

3.2.4. Reinigung der Chinone

Der obige Rückstand wird in Chloroform p. a. aufgenommen und an einer Kieselgelsäule (Kieselgel zur Chromatographie 0,05—0,24mm; Merck) mit Chloroform p. a. als Elutionsmittel gereinigt. Mit dem Eluat der gelben Zone, das das gesuchte Chinon darstellt, wird die chromatographische Reinigung einige Male wiederholt, dann läßt man das Chloroform über Nacht abdunsten und erhält das Methoxychinon als gelbe, amorphe Masse; aus Petroläther umkristallisiert, im Hochvakuum bei 40° sublimiert und erneut aus Petroläther umkristallisiert, liefert sie zartgelbe Nadeln.

Die Eluate der übrigen Zone geben nach dem Ver- dunklen des Chloriform schmierige, braune Rückstände, die auf die Farbteste für Chinone negativ reagieren (Chinhydrone; Additionsprodukte von Diazomethan an Oxychinone) s. Tab. 8).

Ausbeuten: 260—420 mg XVIII, 200—260 mg XXII, 180—240 mg XXXIII.

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Effects of Substrate on Heat Inactivation of Taka-amy lase A

GIITI TOMITA and SAM SOON KIM

Institute of Biophysics, Faculty of Agriculture, Kyushu University, Fukuoka (Japan)


Taka-amy lase A is highly protected from heat inactivation and denaturation by the presence of its substrate, starch. The effect of pH on the protective action was studied to find the relation between the protective action and the state of ionization of taka-amy lase A. The separation of protective effect of substrate from that of hydrolysis products was made by digesting starch with taka-amy lase A before incubation to find the difference of protective abilities of substrate and hydrolysis products. The stabilization of the higher order structure of enzyme protein, depending on the state of ionization of enzyme protein, brought about by the formation of enzyme-substrate and -product complexes seems to be responsible for the appearance of the protective effect observed.

Some enzymes, for example, D-amino-acid oxidase ¹, glutamic dehydrogenase ², alcoholic dehydrogenase ³, lactic dehydrogenase ⁴, bacterial α-amylase ⁵ and taka-amy lase A ⁶—⁷, are considerably protected from the inactivation and denaturation by the presence of coenzymes or substrates or related compounds.

Such a phenomenon is of interest not only from the standpoint of the mechanism of inactivation or denaturation, but also from that of the enzymatic reaction mechanism, since a structural change of enzyme protein induced by the presence of coenzymes or substrates or related compounds is thought to be closely related to the appearance of enzymatic activity.

The protective effect of starch on the inactivation and denaturation of taka- and bacterial α-amylase by heat, acid and urea has been studied by OKUNUKI and others ⁴—⁷.

They concluded that the protective effect must be derived from the hydrolysis products of starch, for the reason that starch was completely hydrolysed

¹ K. BURTON, Biochem. J. 48, 458 [1951].
within a very short digestion time. Furthermore, they concluded that the protective effect of digestion products was attributed to protection of the secondary structure of enzyme protein from denaturation.

In the present investigation, the effect of pn on the protective action of substrate from heat inactivation and denaturation of taka-amylase A was studied to find the relation between the protective effect and the state of ionization of taka-amylase A, and the separation of protective effect of starch from that of decomposition products was performed under the suitable experimental conditions to find the difference between the protective abilities of substrate and hydrolysis products at various states of ionization of taka-amylase A.

The possible explanations were given to the results obtained.

**Experimental**

Taka-amylase A was prepared from ‘takadiastase Sankyo’ (a preparation obtained from a wheat bran culture of *Aspergillus oryzae*) and recrystallized three times from aqueous solution containing 0.01 M calcium acetate by the Akabori’s method.

Taka-amylase A solutions were prepared in 0.02 M acetate or veronal or glycine buffers containing potassium chloride added to the final ionic strength of 0.01. The concentration of taka-amylase A was determined spectroscopically assuming the extinction of taka-amylase A in acetate buffer (pH = 5.6; µ = 0.1) to be $E_{1\%}^{\text{cm}} = 22.1$ at 278.5 nm.

The heat treatment for inactivation and denaturation was carried out as follows: aqueous solutions of taka-amylase A at various pH values, in the absence and presence of soluble starch, were incubated for 10 min at various temperatures in the thermostat and rapidly cooled at 0 °C after incubation.

In order to separate roughly the protective effect of starch from that of hydrolysis products, the digestion of starch with taka-amylase A was made at 0 °C for various periods before incubation.

The activity of taka-amylase A was measured at 40 °C by the blue value method.

Other procedures will be described in the following section.

Chemicals employed were of special grade for analytical use.

**Results and Discussion**

Taka-amylase A is highly protected from heat inactivation by the presence of its substrate, starch.

This is shown in Fig. 1. Curves 1 and 2 show activity-incubation temperature relation in the absence and presence of starch, respectively. The incubation was made for 10 min at pH 5.6 and the activity was measured at pH 5.6, the optimum pH.

In the absence of substrate, activity decreases suddenly over 50 °C and is completely lost at about 65 °C.

On the other hand, the presence of substrate (6 mg/ml of starch) gives rise to a remarkable resistance against heat inactivation, and the activity-incubation temperature curve shifts towards higher incubation temperature than curve 1.

The amount of this shift is about 3.5 °C under the present experimental conditions, and this is thought to be “an effective temperature” lowered as substrate effect. Curve 3 is the difference between curves 1 and 2.

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Fig. 2 shows the dependence of activity on incubation time at an incubation temperature of 60 °C, which is the temperature with the most effective protective action. Curves 1 and 2 show activity-incubation time relation in the absence and presence of substrate, respectively. It takes a much longer incubation time in the latter case than in the former to obtain an equal degree of inactivation. This is
shown in curve 4 obtained from the difference between the incubation time in curves 1 and 2 at the same inactivation. From the extrapolation of curve 4 to zero activity, it is known that the longer incubation time by about 9 min is necessary in the coexistence system of substrate than in the substrate-free system for complete inactivation. From the extrapolation of curve 4 to zero activity, it is known that the longer incubation time by about 9 min is necessary in the coexistence system of substrate than in the substrate-free system for complete inactivation. Curve 3 is the difference between the activity in both systems at the same incubation time. The optimum activity is observed after about 9 min of incubation.

The presence of substrate yields the protective action from heat inactivation not only at the optimum $p_H$ but also in wide $p_H$ regions. This is shown in Fig. 3. Curves 1, 2 and 3, and a, b and c show the dependence of activity of heat-treated taka-amylase A, measured at the $p_H$ values given, on $p_H$ in the absence and presence of substrate, respectively. The activity of non-heat-treated taka-amylase A, measured at the $p_H$ values given, also depends strongly on $p_H$, showing a slight decrease below $p_H$ 5.6 and a profound decrease in the $p_H$ region 5.6—8.0, as seen in Fig. 3, curve 0. Above $p_H$ 8.0, the enzymatic activity completely vanishes. Therefore, the curves mentioned above (1, 2, 3 and a, b, c) are thought to show the combined effects of heat and $p_H$ inactivations. Curve 0' is the activity of non-heat-treated taka-amylase A measured at the optimum $p_H$ (5.6) after exposure overnight to the $p_H$ values given at 20 °C. The inactivation is completely reversible up to about $p_H$ 10.5. Above $p_H$ 10.5, however, the $p_H$ inactivation is irreversible due to an irreversible structural change of taka-amylase A molecule.

From measurements of ultraviolet absorption, optical rotation and enzymatic activity as a function of $p_H$, Takagi and Isemura have found that the most of the phenolic hydroxyl groups in taka-amylase A are not freely ionizable but ionize irreversibly above $p_H$ 10.5 and the native structure of taka-amylase A does not change below $p_H$ 10.5 but above this $p_H$ it is degraded by the irreversible ionization of the phenolic hydroxyl groups which may be hydrogen-bonded to carboxylate groups and may play a part in maintaining the stable structure of enzyme protein.

On the other hand, the heat inactivation is irreversible in the whole $p_H$ regions as in the cases of ordinary enzymes.

In order to eliminate the irreversible part of $p_H$ inactivation, the activity of taka-amylase A heat-treated at various $p_H$ values was measured at $p_H$ 5.6. Curves 1', 2', and 3', and a', b' and c' show the activities, measured at $p_H$ 5.6, of taka-amylase A heat-inactivated at the $p_H$ values given in the absence and presence of starch, respectively.

* A molecule of taka-amylase A has 28 phenolic hydroxyl groups, of which 20 groups are considered to be hydrogen-bonded to the carboxylate groups and masked. cf. Reference 9 and T. Takagi and T. Isemura, J. Biochemistry [Tokyo] 49, 43 [1961].
The heat inactivation is minimum at about $p_H$ 7.0, and steeply increases with decreasing $p_H$ in acidic regions and gradually with increasing $p_H$ in alkaline regions. Above about $p_H$ 10.5, the heat inactivation shows a remarkable increase as the result of the combined effects of heat inactivation and irreversible $p_H$ inactivation.

From the curves (1', 2', 3' and a', b', c', and 0') in Fig. 3, the degree of protection by substrate from heat inactivation defined by (difference between the activity decrease by heat-treatment in the absence and presence of starch)/(activity decrease by heat-treatment in the absence of starch) is obtained at various $p_H$ values, for various incubation temperatures and plotted in Fig. 4. The values of degree of protection for the incubation temperatures given fall roughly on a curve which shows a slight decrease with increasing $p_H$ in the region $p_H$ 6—10, and the sudden decrease in both sides below $p_H$ 6 and above $p_H$ 10. A slight decrease of protective action in the range $p_H$ 6—10 may be due to the changes in calcium binding properties in this region, as reported by Katz and Klotz in bovine serum albumin. The decrease of protective action in the acidic and extreme alkaline regions suggests that the protective ability is weakened by the reversible ionization of carboxylate or phenolic hydroxylate groups of carboxylate-phenolic hydroxylate hydrogen bonds playing an important part in stabilizing the native structure of taka-amylase A and the protective ability is completely lost by the irreversible ionization of these groups.

The protective action observed in the presence of starch is thought to be derived from the superimposed effects of starch and its hydrolysis products. The separation of protective effect of starch from that of hydrolysis products was made by digesting starch for various periods at 0 °C before incubation. The dependence of activity on digestion time is shown in Fig. 5, curve 1. The incubation temperature and time are, respectively 59 °C and 10 min, at which the protective action is most remarkable, as shown in Figs. 1 and 2.

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In the absence of starch, activity decreases to 32% after heat treatment. The amount of substrate (degree of polymerization $n > ~6$) in the course of digestion, which was measured by iodostarch reaction, is shown in Fig. 5, curve 2.

As shown in Fig. 6, the decrease in activity by heat treatment is proportional to the concentration of substrate in the region of low substrate concent-

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12 The minimum degree of polymerization of amylose to colour on treatment with iodine. cf. M. A. Swanson, J. Biol. Chemistry 172, 825 [1948].
tration, where the incubation temperature and time are, respectively, 60 °C and 10 min and incubation was started immediately after mixing the substrate with amylase solution. This proportionality makes it possible roughly to separate the total protected amount (expressed in activity difference between curve 1 and the activity after heat treatment in the absence of substrate) into two parts: protected amount by substrate \((n>6)\) and that by decomposed compounds \((n<6)\). These are shown in Fig. 5, curves 3 and 4, respectively.

In the short period of digestion, the protection is mainly due to the effect of substrate \((n>6)\). The decomposition products from starch, \(\alpha\)-limit dextrin and maltose are also effective for protection of amylase from heat inactivation, but glucose has no protective effect. In the presence of these substances (6 mg/ml each), the activity decreases by heat treatment (at 59 °C and for 10 min) to 38, 37 and 32%, respectively, as shown in Fig. 5. Limit dextrin and maltose seem to stabilize the secondary structure by combination with amylase, and to protect it from heat inactivation and denaturation, though the effect is not so great.

The dependence of protected amount – difference between activities (measured at \(p_H\) 5.6) of amylase heat-treated in the absence and presence of starch – on digestion time before incubation is shown in Fig. 7. The protected amount at zero digestion time is normalized to unity. Curves 1 and 2, and a and b, are the protected amounts when incubated for 10 min at \(p_H\) 5.6 and 9.0, respectively. The amount of substrate in the course of digestion, which was measured by iodostarch reaction, is shown in Fig. 7, curve 3. Curves 3 and 1', 2', a', b' are the amount protected by starch and those by decomposition products, respectively. The proportion of protection by digestion products to that by starch at \(p_H\) 5.6 is larger than that at \(p_H\) 9.0. This means that digestion products are less effective for the protection from heat inactivation of taka-amylose A in \(p_H\)-inactivated or -denatured states.

According to LEVITZKI and others\(^{13}\), pancreatic \(\alpha\)-amylase can form an insoluble complex with macro-dextrin. The yield of insoluble complex is dependent on the size of dextrin and the optimal precipitation occurs only within a limited range of the enzyme/dextrin ratio. Excess amylase or excess dextrin inhibits completely precipitation of complex. Taka-amylose A can also form at 0 °C an insoluble and stable complex with starch within a limited range of enzyme/substrate ratio, as shown in Fig. 8. The yield of enzyme precipitated by the formation of insoluble complex was calculated from the amount of enzyme protein left in the supernatant after the mixture of taka-amylose A and starch was centrifuged in the cold.

\(^{13}\) A. LEVITZKI, J. HELLER, and M. SCHRAM, Biochim. biophysica Acta [Amsterdam] 81, 101 [1964].
Fig. 8. Effect of starch concentration on formation of insoluble complex. Concentration of taka-amylase A, 0.29 mg/ml. Solutions of taka-amylase A and starch at pH 8.0 were mixed at 0 °C.

Under the present experimental conditions of heat inactivation, the taka-amylase A/starch ratio is too small to yield precipitation of complex. In such an excess-substrate zone, the formation of insoluble complex is prevented and only soluble complex is formed. Therefore, the insoluble complex does not take part in the protective action observed in the present investigation.

The destruction of enzyme structure caused by heat treatment leads to inactivation and denaturation, and makes it possible for denatured enzyme molecules to combine to aggregates. For this reason, the taka-amylase A solution becomes turbid by the occurrence of heat denaturation. The turbidity is a measure of aggregation of denatured enzyme molecules. The turbidity due to heat denaturation was measured by the optical density at 500 nm and is shown in Fig. 9. The turbidity runs parallel to the inactivation and is inhibited by the presence of substrate. From these results, it is considered that the formation of enzyme-substrate and -product complexes not only highly stabilizes the higher order structure of taka-amylase A molecule, but prevents the formation of aggregation of enzyme molecules induced by heat denaturation.

The dependence of turbidity of heat-treated taka-amylase A solutions, in the absence and presence of starch, on pH is shown in Fig. 10. In alkaline region,

Fig. 9. Effect of incubation temperature on turbidity and activity. Curves 1 and a, the activity of heat-treated taka-amylase A in the absence and presence of starch, respectively; concentration of taka-amylase A, 0.036 mg/ml; concentration of starch, 6 mg/ml. Curves 2 and b, the turbidity measured by the optical density at 500 nm in the absence and presence of starch, respectively; concentration of taka-amylase A, 0.089 mg/ml; concentration of starch, 7.5 mg/ml. The heat-treatment was made for 10 min at pH 6.0 °C.

Fig. 10. Effect of pH on turbidity. Curves 1 and a, the turbidity measured by the optical density at 500 nm in the absence and presence of starch, respectively; curve 2, difference between curves 1 and a; concentration of taka-amylase A, 0.089 mg/ml; concentration of starch, 7.5 mg/ml. The heat-treatment was made for 10 min at 60 °C.

15 L. Michaelis and M. L. Menton, Biochem. Z. 49, 333 [1913].
17 K. G. Stern, J. Biol. Chemistry 114, 437 [1939].
19 D. G. Doherty and F. Vaslow, J. Amer. Chem. Soc. 74, 931 [1952].
shown spectroscopically the existence of complexes of peroxides with catalase and peroxidase and calculated the thermodynamic quantities of enzyme-substrate complex such as free energy, entropy and enthalpy.

Recently, the change of higher order structure of enzyme on the formation of enzyme-substrate complex has been investigated by means of optical rotation and difference spectrum. These experimental results show that the structure of enzyme protein changes from disorder to order on the formation of enzyme-substrate complex. Such a structural change is thought not only to conjugate intimately with the appearance of enzymatic activity in enzyme-catalyzed reaction, but also to play an important role in the protective action of substrate from heat inactivation and denaturation.

A difference spectrum of taka-amylase A has not been observed, but, in the cases of lysozyme and \( \alpha \)-chymotrypsin\(^{20-23} \), difference spectra on the formation of enzyme-substrate complex are well established. On the formation of enzyme-substrate complex, ultraviolet absorption spectra of enzymes usually show “red shift” and this means the conformation change of enzyme protein from disorder to order. This conformation change may conjugate with the appearance of enzymatic activity. The ordered and rigid structure of enzyme protein may be essentially responsible for enzymatic activity. Furthermore, this structure may be highly stabilized and be protected from external disturbance such as thermal agitation. This seems to explain why taka-amylase A can be protected by the presence of its substrate.

The presence of hydrolysis products also stabilizes the higher order structure of enzyme protein by the formation of enzyme-product complex and protects enzyme from heat inactivation.

As suggested by Isemura and other\(^{26} \), the site with which decomposition compounds are combined, unlike substrate, is not necessarily the active point of enzyme. Therefore, the induced stabilization responsible for the protective action is considered to be probably similar to that by calcium ions.

The protective action appears in the limited \( p_H \) region where the carboxylate and phenolic hydroxylate groups ionize reversibly and reversible conformation change can occurs and \( p_H \) inactivation is reversible. However, it can not be observed in the extreme acidic and alkaline regions where the irreversible conformation change leading to the irreversible \( p_H \) denaturation occurs.

In this connection, it is considered that there is an intimate relation between the induction of the protective effect and the state of ionization of enzyme protein.

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