Protective Action of Substrate from Heat Inactivation of Taka-Amylase A

It is known that some enzymes[1–5] are protected from inactivation and denaturation by the presence of co-enzymes or substrates or related compounds.

TOMITA and KIM[6] have dealt with the protective action of a substrate, starch, from heat inactivation of taka-amylase A (TAA) and have tried to separate the protective effect of starch from that of its decomposition products under suitable experimental conditions.

In the present investigation, the effect of pH on the protective action of substrate from heat inactivation and denaturation of TAA was studied to find the relation between the protective effect and the state of ionization of TAA and to find the difference between the protective abilities of substrate and hydrolysis products at various states of ionization of TAA.

TAA begins to be inactivated by heat at about 50 °C. However, when its substrate is present, it can be highly protected from heat inactivation in wide pH regions, as seen in Figure 1. Curves 1, 2 and 3, and a, b and c show the dependence of activity of heat-treated TAA, measured at the pH values given, on pH in the absence and presence of starch, respectively.

Heat-treatment was carried out by the following procedure. Aqueous solutions of TAA (0.0036%) at various pH values, in the absence and presence of 0.6% soluble starch, were incubated for 10 min at various temperatures and rapidly cooled to 0 °C after incubation. The activity of TAA was measured at 40 °C by the blue value method[7].

The activity of non-heat-treated native TAA also depends strongly on pH, showing a slight decrease below pH 5.6 and a profound decrease in the pH region 5.6–8.0, as seen in Figure 1, curve 0. Above pH 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 pH inactivations. Curve 0′ is the activity of non-heat-treated TAA measured at the optimum pH (5.6) after exposure overnight to the pH values given at 20 °C. The inactivation is completely reversible up to about pH 10.5. Above pH 10.5, however, the pH inactivation is irreversible due to a structural change of enzyme protein induced by the irreversible ionization of masked phenolic hydroxyl groups which may be hydrogen-bonded to carboxylate groups and may play a part in maintaining the native structure[8].

Curves 1′, 2′ and 3′ and a′, b′ and c′ show the activities, measured at pH 5.6, of TAA heat-inactivated at the pH values given in the absence and presence of starch, respectively. The heat inactivation is minimum at about pH 7.0, and steeply increases with decreasing pH in acidic regions and gradually with increasing pH in alkaline regions. Above about pH 10.5, the heat inactivation shows a remarkable increase as the result of the combined effects of heat inactivation and irreversible pH inactivation.

The conformation change induced by the formation of an intermediate enzyme-substrate complex, which is thought to conjugate intimately with the appearance of enzyme activity in enzyme-catalysed reactions, may lead to stabilization of the secondary structure of enzyme protein and to protection from external disturbances, for example, by heat treatment. This seems to explain why TAA can be protected by the presence of starch.

The protective effect observed is thought to be derived from the superimposed effects of starch and its hydrolysis products. The separation of the protective effect of starch from that of its decomposition products is possible if the incubation is made after various periods of digestion.

The dependence of the protected amount – the difference between activities (measured at pH 5.6) of TAA heat-treated in the absence and presence of starch – on digestion time before incubation, is shown in Figure 2. Curves 1 and 2 and a and b are the protected amounts when incubated for 10 min at pH 5.6 and 9.0, respectively. The amount of substrate in the course of digestion, which was measured by iodostarch reaction, is shown in Figure 2, curve 3. Since the protected amount is proportional to the concentration of substrate in the region of substrate

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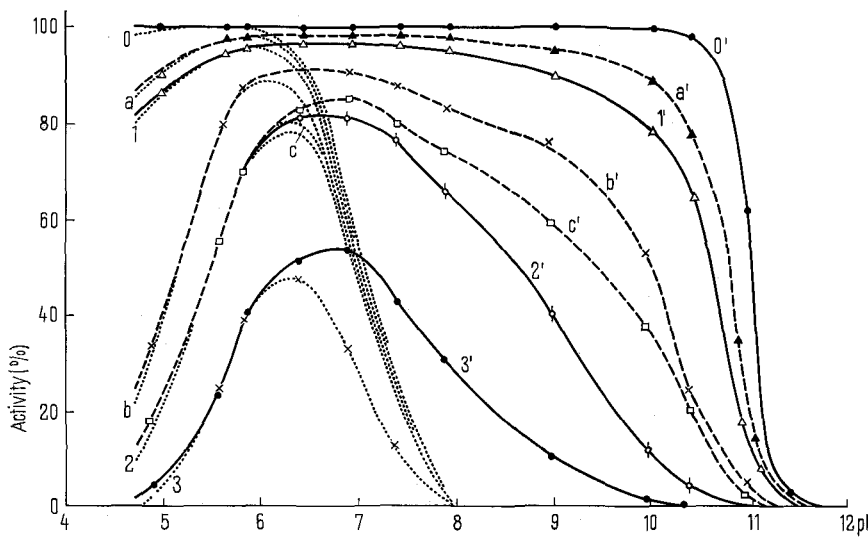

Fig. 1. Effect of pH on activity of taka-amylase A (TAA). The activity of non-heat-treated TAA: curves 0 and 0′, measured at the pH values given and at pH 5.6 after overnight treatment at the pH values given at 20 °C, respectively. The activity of TAA heat-treated in the absence of starch at the pH values given: curves 1, 2 and 3, measured at the pH values given; curves 1′, 2′ and 3′, measured at pH 5.6. The activity of TAA heat-treated in the presence of starch at the pH values given: curves a, b and c, measured at the pH values given; curves a′, b′ and c′, measured at pH 5.6. The heat-treatment was made at 50, 55 and 60 °C respectively for 10 min. Concentration of TAA and starch, 0.036 and 6 mg/ml, respectively. TAA solutions were prepared in 0.02 *M* acetate, veronal or glycine buffers containing potassium chloride added to the final ionic strength of 0.01.

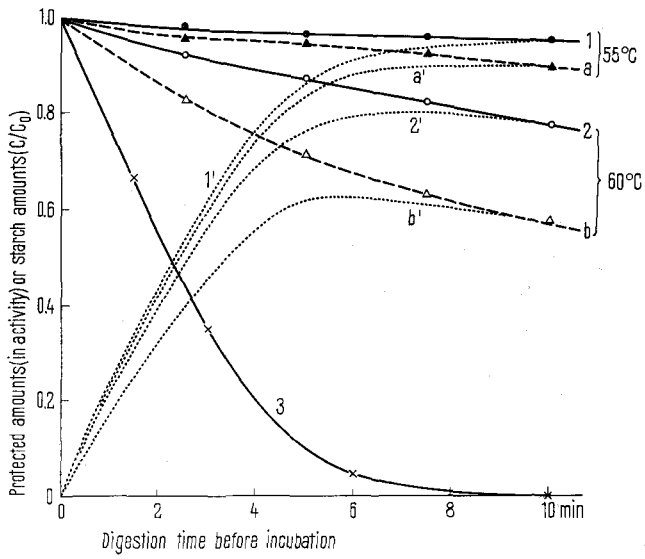

Fig. 2. Changes of protected amounts (difference between activities of TAA heat-treated in the absence and presence of starch) with digestion time before heat-treatment. Curves 1, 2, and a, b, heat-treated at pH 5.6 and 9.0, respectively; curve 3, starch concentrations at various digestion times before incubation, and also means the amount protected by starch; curves 1′, 2′, and a′, b′, amounts protected by decomposition products from starch, corresponding to the cases of curves 1, 2, and a, b, respectively. The digestion was made at pH 7.0 at 20 °C in all cases. The heat-treatment was made for 10 min at the temperature shown and at pH 5.6 or 9.0, immediately after digestion. The protected amount at zero digestion time is normalized to unity. The activity was measured at pH 5.6. Concentrations of TAA and starch, 0.036 and 6 mg/ml, respectively.

concentrations employed in the present investigation, it is possible roughly to separate the total protected amount into two parts: the amount protected by starch and that protected by decomposition products. These are shown in Figure 2, curves 3 and 1′ or 2′, or a′ or b′ respectively. In the short period of digestion, the protection is mainly due to the starch effect. The proportion of protection by digestion products to that by starch at pH 5.6 is larger than that at pH 9.0, as seen in Figure 2. This means that digestion products are less effective for the protection from heat inactivation of TAA in pH-inactivated or -denatured states.

Further details will be published in a future paper.

Zusammenfassung. Nachweis eines Schutzes der Eiweissstruktur, der Taka-Amylase A, gegen Wärme-Inaktivierung und -Denaturierung durch das Substrat. Auf Grund der pH-Abhängigkeit dieser Schutzwirkung kann angenommen werden, dass sie die Folge der Stabilisierung der Sekundärstruktur des Proteins durch Enzym-Substrat- und -Produkt-Komplexe ist.

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Inhibition of Skin Calcification (Calciphylaxis) by Polyphosphates

Condensed phosphates, such as inorganic pyrophosphate and long chain polyphosphates (e.g. Graham salt), are able to block collagen-induced calcium phosphate precipitation in vitro in concentrations as low as $10^{-6} M$[1]. Condensed phosphates can also prevent the mineralization of chick embryo femurs grown in tissue culture[2]. These facts, together with the presence of pyrophosphate in both urine[3] and plasma[4] suggest that pyrophosphate may play an important role in the biological regulation of calcification in vivo. Recently it has been shown that pyrophosphate and long chain polyphosphates can indeed inhibit calcification in vivo, as they are able to block vitamin D_3-induced aortic calcification in rats[5].

In the present study we have investigated the inhibitory effect of Graham salt on another calcification system in vivo. As our model of calcification, the calciphylactic skin reaction described by Selye[6] was chosen. Graham salt was used as the condensed phosphate because pyrophosphate was found to cause skin necrosis at the site of injection.

Material and methods. Female Wistar rats weighing about 130 g were given Altromin-R rat diet (Altromin, Lage, Germany) and tap water ad libitum throughout the experiment. 42 rats were allotted to 5 groups as follows: Group I: a single dose (10 mg/kg) of dihydrotachysterol (DHT), obtained from Wander AG, Berne, Switzerland, was dissolved in arachis oil and given by stomach tube. 24 h later epilation was performed under ether anaesthesia on an area of skin of 9 cm² in the intrascapular region. On the 7th day after DHT the animals were killed with ether. Group II: similar to group I, but the animals were in addition given daily subcutaneous injections of Graham salt (J. A. Benckiser, Ludwigshafen a. Rh., Germany) at a dose of 1 mg P/kg/day, beginning 2 days before the administration of DHT and continuing throughout the course of the experiment. The Graham salt was

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