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PREPARATION OF CONJUGATES OF α-AMYLASE WITH A PROTEASE INHIBITOR AND THEIR STABILITY

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A method for obtaining conjugates of α-amylase with a trypsin inhibitor and separating them into fractions has been developed. Two fractions have been obtained — thermostable and thermolabile. The thermostable fraction retained about 80% of its amylase activity after incubation at 50°C for 2 h, with activation of the enzyme during the first 30 min. In the presence of trypsin the conjugated enzyme retained 91% of its initial activity after incubation for 1 h, although the activity of the native enzyme fell to 35% under the same conditions.

We have previously [1, 2] developed a simple method for eliminating proteases from enzyme preparations that leads to a rise in the activity of the enzymes and also to an increase in their stability under the action of heat and environmental factors [3]..At the same time, the problem of the antiproteolytic protection of enzymes also arises under the conditions of their employment. Being components of many biological fluids, proteases act adversely on any biocatalysts used [4]. This appears particularly when enzymes are employed for analytical purposes: proteases lower the quality of enzyme electrodes and affect their stability characteristics; in the performance of immunoenzyme assays they distort the results to an uncontrollable degree.

We have set ourselves the task of ensuring the antitrypsin protection of enzymes by the covalent conjugation, through glutaraldehyde, of α-amylase with a trypsin inhibitor, which was assumed to protect the enzyme from proteolysis. Conjugation was achieved in solution under conditions analogous to those selected for the conjugation of α-amylase with antibodies [3].

Since binding took place at the amino groups of the proteins and was nonspecific, conjugation could have led to conjugates with different ratios of the molecules of the amylase and of the trypsin inhibitor. It is obvious that this could profoundly affect the properties of the conjugates. We therefore fractionated the conjugates obtained on Sephadex G-75.

As can be seen from Fig. 1*a*, the curves of the protein and of the activity of the trypsin inhibitor (*1* and *3*) repeated one another in shape. The fractions possessing trypsin inhibitor activity issued from the column in two peaks. A fraction with the conjugated inhibitor possessing a high molecular mass was probably eluted first and then a fraction with the inhibitor in the free form.

The fractions possessing α-amylase activity were eluted from the column in a single peak, but the compound corresponding to this peak was eluted from the column earlier than pure amylase when the individual enzyme was chromatographed in the second variant (Fig. 1*b*), and, consequently, it may be concluded that the bulk of the amylase was in a conjugated state but with different amounts of bound inhibitor. Issuing from the column faster was a fraction of conjugate more highly loaded with the trypsin inhibitor (in Fig. 1*a*, the hatched region *I*), then a fraction with a low inhibitor content (hatched region *II*).

The thermostabilities of the two fractions of conjugate were investigated separately. Figure 2 shows the results of a determination of the thermostabilities at 50°C of the initial enzyme and of the two fractions of conjugate. The first fraction (Fig. 2, curve *2*), containing a larger amount of inhibitor molecules and smaller amount of amylase, proved to be more thermostable than the initial enzyme (Fig. 2, curve *1*). Thus, after incubation at 50°C for two and a half hours the conjugate had retained about 80% of its initial activity, while the native enzyme had been almost half inactivated. Furthermore, activation of the amylase was observed during the first 30 min of incubation of the conjugate at 50°C, after which the activity of the enzyme reached its initial level and began to fall.

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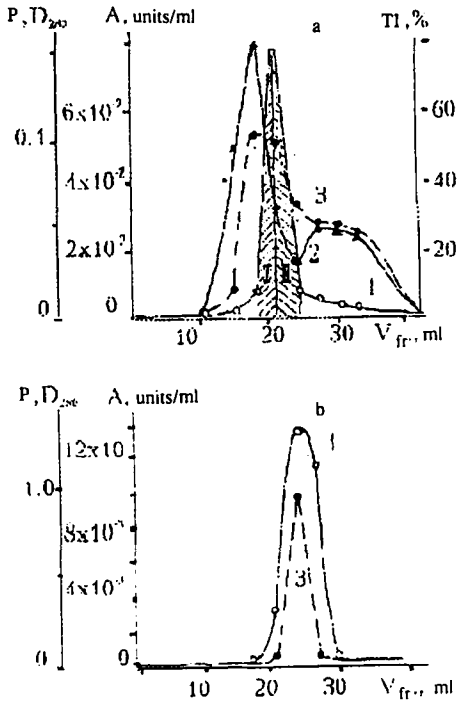

Fig. 1. Gel filtration of α-amylase through Sephadex G-75: *a*) α-amylase conjugate with trypsin inhibitor; *b*) native α-amylase; *1*) α-amylase activity; *2*) trypsin-inhibiting activity; *3*) protein; *A*) activity; *B*) protein; *TI*) trypsin inhibitor.

The second fraction of the conjugates, containing fewer inhibitor molecules and more amylase proved to be thermolabile. Its activity at 50°C after incubation for more than 90 min was even lower than that of the initial enzyme (Fig. 2, curve *3*).

In view of this, in the following experiment we investigated only the first, thermostable, fraction of the conjugate. The results so obtained are shown in Table 1, from which it can be seen that by conjugating the enzyme with the trypsin inhibitor it was possible to raise the resistance of α-amylase to proteolysis substantially. Thus, while after the native enzyme had been incubated for an hour in the presence of trypsin about 35% of the initial activity was retained, in the conjugated state the degree of retention was about 91%.

Thus, it has been possible to obtain a heat-stable fraction of a conjugate of α-amylase with the trypsin inhibitor that exhibits substantial protection from proteolysis.

EXPERIMENTAL

We used purified α-amylase from the preparation amilorizin P10x [2] and soybean trypsin inhibitor from Reanal, Hungary.

α-Amylase activity was determined by a standard method [5] and is expressed in grams of starch hydrolyzed in one minute by one gram of enzyme or one milliliter of enzyme solution. Protein was determined spectrophotometrically. The conjugates were obtained in a solution containing 0.25 ml of enzyme solution (1 mg/ml), 0.25 ml of trypsin inhibitor solution (0.5 mg/ml), 0.75 ml of borate buffer (0.1 M, pH 8.0), and 0.1 ml of glutaraldehyde (0.01%), with stirring on a magnetic stirrer at room temperature for 20 min. Then the solution was left overnight with constant stirring. The conjugate was purified on a column (1 × 30 cm) of Sephadex G-75.

Thermostability was determined at 50°C. An aqueous solution of enzyme or conjugate was kept in a water bath at the given temperature for predetermined times and was then cooled, and the activity was determined under standard conditions [5].

TABLE 1. Stability of α-Amylase in the Presence of Trypsin

Activity	Native enzyme		
	initial, units/g	after 1h incubation, units/g	residual activity after 1h, %
Without trypsin	$0.80.10^3$	$0.75.10^3$	94
In the presence of trypsin	$1.00.10^3$	$0.35.10^3$	35
	Conjugate of α-amylase with trypsin inhibitor (heat-stable fraction)		
	initial, units/g	after 1h incubation, units/g	residual activity after 1h, %
Without trypsin	$0.47.10^3$	$0.60.10^3$	127
In the presence of trypsin	$0.47.10^3$	$0.43.10^3$	91

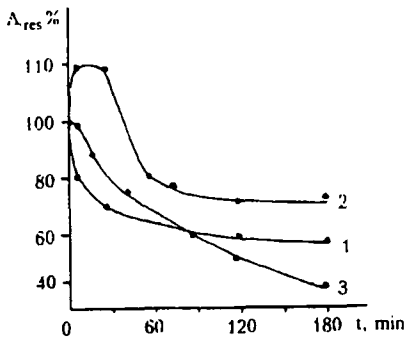

Fig. 2. Heat stability of α-amylase preparations at 50°C: *1*) native enzyme; *2*) conjugate of α-amylase with trypsin inhibitor, fraction I; *3*) conjugate of α-amylase with trypsin inhibitor, fraction II.

To determine resistance to proteolytic decomposition, the solution or conjugate was kept in the presence of trypsin (0.4 mg/ml) for an hour, and then the activity was determined under the standard conditions and compared with the initial value.

REFERENCES

1. M. M. Rakhimov and Kh. T. Khasanov, Biotekhnologiya, **5**, No. 2, 189 (1989).
2. Sh. S. Tashmukhamedova, Kh. T. Khasanov, M. M. Rakhimov, M. S. Tashmukhamedov, and Sh. I. Salikhov, Zh. Prikl. Biokhim. Mikrobiol., **31**, No. 3, 272 (1995).
3. Sh. S. Tashmukhamedova, Author's Abstract of Dissertation ... Candidate of Biological Sciences [in Russian], Tashkent (1986).
4. M. M. Rakhimov, The Use of Enzyme Preparations in the Food Industry of Uzbekistan [in Russian], Tashkent (1983), p. 62.
5. Enzyme Preparations. Method of Determining Amylolytic Activity [in Russian], GOST [State Standard] 20269, Moscow (1975).