1,2,7,8-Diphthaloylcarbazol-Gehalt durch Kolorimetrieren der Lösung einer Probe in konz. Schwefelsäure bei λ 526 nm bestimmt.

Analog wurde auch bei den übrigen Versuchen unter den im Text diskutierten Bedingungen verfahren.

Für technische Mitarbeit danken wir Frau L. Musilová.

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STABILITY OF ALKALINE PROTEASE FROM *Aspergillus flavus*

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The serine alkaline protease[1,2] from the mold *Aspergillus flavus* is very unstable in the absence of salts. The desalted preparations of the protease kept at −20°C were autolyzed already after a few weeks. In this characteristic suceptibility to rapid autolysis the alkaline protease resembles among others, trypsin[3] and subtilisin[4,5]. In this study, the effect of pH, temperature, metal ions, and certain other agents on the stability of the alkaline protease is investigated and the process of its inactivation is compared with the inactivation of trypsin and subtilisin.

EXPERIMENTAL

Material: The alkaline protease was prepared by the procedure described by us[1] from the crude proteolytic preparation of the mold *Aspergillus flavus* produced in the Research Institute of Food Technology, Czech Academy of Agriculture, Prague. Hemoglobin was a commercial preparation of Zdravotnické potřeby, Prague, the other chemicals were purchased from Lachema, Brno.

The measurement of proteolytic activity was carried out by the method of Anson[6] using a solution of denatured hemoglobin. To 2 ml of the hemoglobin solution at pH 7 (Britton–Robinson

buffer) at 37°C, 0·2 ml of the protease solution was added. The mixture was incubated 10 min at 37°C and the reaction discontinued by the addition of 5 ml of 5% trichloroacetic acid. The precipitate was filtered off and the absorbance of the filtrate was measured at 280 nm.

Effect of pH *on proteolytic activity* of the alkaline protease. To 2 ml of the Britton–Robinson buffer at various pH (*cf.* Fig. 1) 0·1 ml of the solution containing 0·3 mg of the alkaline protease was added. The mixture was set aside at room temperature (25°C) and the proteolytic activity was measured at intervals of 10 min, $2^1/_2$ h, and 22 h.

Effect of temperature on proteolytic activity of the alkaline protease. After 30 min incubation of 0·025% solution of the alkaline protease in water (pH 5·8), 0·01N-$CaCl_2$ (pH 6·7), and 0·01N-NaCl (pH 6·4) at temperatures given in Fig. 2 the proteolytic activity was determined.

Effect of cations on proteolytic activity of alkaline protease. An 0·01% solution of the protease in 0·1M Tris-HCl buffer, pH 7, was incubated in the presence of cations (concentration 0·01M and 0·001M, respectively) for 10 min at 37°C. After this period the proteolytic activity was determined. The results are summarized in Table I and expressed in per cent of proteolytic activity. The activity of 0·01% solution of the protease in 0·1M-Tris-HCl buffer, pH 7, after the incubation in the absence of metal ions was taken to represent 100%.

Effect of anions on proteolytic activity of alkaline protease. After 0, 1/2, and 3 h incubation of 0·02% solution of the protease at 37°C in 0·1N solutions of sodium salts (*cf.* Table II) at pH 5·3 the proteolytic activity was measured. The results are summarized in Table II and expressed in per cent of proteolytic activity. The activity of 0·02% aqueous solution of the protease (pH 5·3) was taken to represent 100%.

Effect of urea and guanidine hydrochloride on proteolytic activity of alkaline protease. After 30 min incubation of 0·05% solution of the protease in water and in solutions of urea and guanidine hydrochloride (at concentrations given in Fig. 3) at room temperature (28°C) the proteolytic activity was determined.

TABLE I

Effect of Cations on Activity of Alkaline Protease from *A. flavus*

Salts	Relative activity, %		Salts	Relative activity, %	
	0·01M	0·001M		0·01M	0·001M
—	100	100	$PbCl_2$	85	98
NaCl	112	110	$CoCl_2$	80	100
$CaCl_2$	106	102	$ZnCl_2$	58	98
$MgCl_2.6\,H_2O$	102	103	$CdCl_2.2^1/_2\,H_2O$	58	90
Ba acetate	98	99	$HgCl_2$	53	95
KCl	98	100	Ni acetate	38	90
$MnCl_2$	96	97	Cu acetate	34	80
NH_4Cl	96	98			

TABLE II

Effect of Anions on Activity of Alkaline Protease from *A. flavus*

Sodium salt	Relative activity after incubation, %		
	0 h	$^1/_2$ h	3 h
—	100	82	78
Chloride	103	98	99
Borate	99	99	100
Sulfate	97	99	98
Fluoride	97	96	97
Phosphate	97	96	94
Citrate	97	96	94
Acetate	96	96	96

Comparison of inactivation of alkaline protease and trypsin. The residual proteolytic activity of 0·01% and 0·1% solutions of the compared protease in phosphates buffer at pH 7·5 and 37°C was measured as a function of time in parallel experiments. The results are given in Fig. 4.

RESULTS AND DISCUSSION

The alkaline protease is stable in buffer solutions in the pH-range 5—10 (Fig. 1). At both acidic and alkaline pH-values it is irreversibly inactivated. The protease is rapidly inactivated at temperatures over 50°C (Fig. 2). The presence of salts is without any substantial effect on the thermal inactivation.

The protease is inactivated by Cu^{2+}-, Ni^{2+}-, Hg^{2+}-, Cd^{2+}-, Zn^{2+}-, Co^{2+}-, and Pb^{2+}- ions. The presence of K^+-, Na^+-, NH_4^+-, Mn^{2+}-, Mg^{2+}-, Ca^{2+}-, and Ba^{2+}- ions is without effect or has a slight stimulating effect (Table I). By contrast, the anions tested here are without any specific effect (Table II). Compared to the aqueous solution not containing salts, all of the sodium salts tested here stabilize the protease. The protease does not lose its activity after dissolving in 8M urea, is, however, irreversibility inactivated in 4M guanidine hydrochloride (Fig. 3).

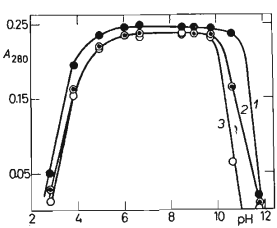

FIG. 1

Relation between pH and Stability of Alkaline Protease from *A. flavus*

The activity was measured at the given pH *1* after 10 min, *2* after $2^1/_2$ h, *3* after 22 h. A_{280} proteolytic activity according to Anson.

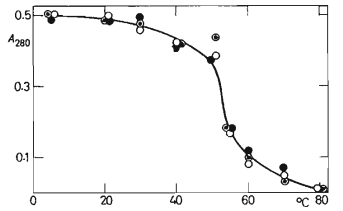

FIG. 2

Relation between Temperature and Stability of Alkaline Protease from *A. flavus*

A 0·025% solution of the protease was thermostated in water (—○—), in 0·01M-NaCl (—●—) and in 0·01M-$CaCl_2$ (—⊙—). A_{280} proteolytic activity according to Anson.

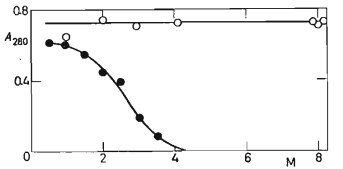

FIG. 3

Effect of Urea and Guanidine Hydrochloride on Activity of Alkaline Protease from *A. flavus*

A 0·05% solution of the protease after the incubation in the solution of urea —○— and guanidine hydrochloride —●— A_{280} proteolytic activity according to Anson.

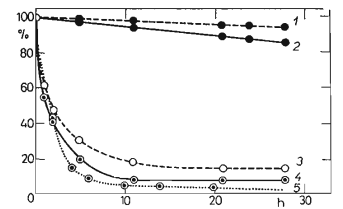

FIG. 4

Inactivation of Alkaline Protease from *A. flavus* and of Trypsin at 37°C in 0·05M Phosphate Buffer at pH 7·5

Concentration of solutions: *1* 0·01% and *2* 0·1% alkaline protease, *3* 0·01%, and *4* 0·1% trypsin, *5* inactivation of the alkaline protease (0·25% solution) in the absence of salt according to ref.[7].

As can be seen in Fig. 4, we found an essential difference in the inactivation of the alkaline protease and of trypsin. Trypsin is inactivated during a very short period while the alkaline protease in 0·01% or 0·1% solution retained even after 28 h 94 and 86% respectively of the original activity. The results of more recent experiments with the 0·25% aqueous solution of the protease in the absence of salts (ref.[7]) are also given in Fig. 4. In this case the pH was kept constant at pH 7 by the addition of 0·05N-NaOH in an autotitrator. We can see that in the absence of the buffer the course of the autolysis of the alkaline protease was the same as that of trypsin.

It is known that trypsin can be protected against autolysis at neutral pH-values by the presence of calcium ions. In our experiments calcium ions were not added and the conditions of the comparison experiment were taken from the study of Hagihara and coworkers[5] who had compared trypsin and a bacterial protease, subtilisin, in the same manner. Subtilisin shares many features in common with our protease. First of all, it does not contain any disulfide bonds[8] in its molecule and the amino acid sequence of its serine active center[9] is identical with the sequence Gly.Thr. .Ser$^+$.Met.Ala found by us in the alkaline protease[2]. As in subtilisin[10], histidine[11] is also involved in the mechanism of action of the alkaline protease.

Both subtilisin and our protease are relatively very stable in salt solutions, they are, however, readily inactivated in solutions which do not contain salts. This phenomenon can be accounted for — in analogy to the explanation presented by Hagihara and coworkers[5] — by denaturation, paralleled by rapid autodigestion. Lazdunski and Delaage[3] have similarly assumed that the autodigestion of trypsin involves the digestion of the so-called inactive trypsin by active trypsin. The comparison is especially interesting from this viewpoint if we consider the difference in the amino acid composition of the compared proteases. The molecule of trypsin contains 6 disulfide bonds[12] whereas subtilisin and the alkaline protease do not contain any disulfides. The molecules of proteins containing disulfide bonds can be expected to be more stable. In fact, the opposite is true. Having regard to the behavior in urea and guanidine hydrochloride we can postulate that for the stabilization of the molecule of the alkaline protease are responsible — in addition to hydrogen bonds and hydrofobic interactions — to a considerable degree especially ionic interactions.

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