POLYMORPHISM OF ALKALINE PROTEINASE FROM *Aspergillus flavus**

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The alkaline proteinase from *Aspergillus flavus* undergoes conversion when exposed to higher temperature (45°C, close to the transition temperature) or when treated with 8M urea. The resulting mixture of enzymes can be resolved into two groups by ibn-exchange chromatography on DEAE-Sephadex. The diisopropylphosphoryl derivative of the proteinase does not undergo these changes. The presence of Ca^{2+}-ions or of ε-aminocaproic acid (which stabilize the enzyme) or of ethylenediaminetetraacetic acid (which labilizes the enzyme) during the heating or during the treatment with urea affect the varying forms of enzyme in different ways. These forms of enzymes differ in their pH-optimums of cleavage of hemoglobin or in the specificity of cleavage of the B-chain of oxidized insulin.

In our preceding papers we have described the isolation of the extracellular alkaline proteinase from the mold *Aspergillus flavus*[1], the determination of its serine active center[2], its specificity assayed with the B-chain of oxidized insulin and certain synthetic substrates[3], and kinetic data showing the participation of histidine in the active center of the proteinase[4]. We have also reported the conditions of stability of this enzyme[5], the determination of its molecular weight[6], its comparison with proteinases from other molds of the genus *Aspergillus*[7], as well as preliminary results of sequential studies on fragments of this enzyme[8,10].

When isolating and characterizing the extracellular alkaline proteinase from *Aspergillus flavus* we found that the pH-optimum of proteolytic activity of this enzyme varied within the range pH 7–9 depending on the method of its preparation and on its age[9]. To cast light on these changes we studied the properties which the enzyme acquires at different temperatures.

* A part of this study has been reported elsewhere[8,10].

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EXPERIMENTAL

Material

The alkaline proteinase was prepared from the centrifuged culture medium of the mold *Aspergillus flavus* supplied by the Research Institute of Food Industry, Czechoslovak Academy of Agriculture, Prague. Hemoglobin (Hb)* and all the reagents of G.R. purity grade were purchased from Lachema, Prague. The dialyzing tubing was a product of Kalle, Wiesbaden. ε-Aminocaproic acid was purchased from Ziotolek, Poznań, Poland.

Isolation of alkaline proteinase from A. flavus: In this work we modified the originally described method[1] of preparation of the enzyme. We started with the culture medium (20 l) and according to Lallouette and Bourderon[11] the sorption (at pH 4·5) and desorption (with 1M ammonium acetate pH 5·5) was effected by filtration through 3·5 kg of Amberlite IRC-50 (20—50 mesh). The desorbed product was precipitated with cold acetone (−50°C) and after desalting on Sephadex G 25 equilibrated with 0·01M sodium phosphate pH 5·9, it was chromatographed on DEAE-Sephadex A-50 (Fig. 1). The pooled fractions of the peaks A and B were freed of phosphate ions by gel filtration on Sephadex G-25 equilibrated with ammonium acetate at pH 7. After precipitation with acetone 2—4 ml of the concentrated solution of fraction A (approximately 7—15 μmol of the enzyme) and 1—2 ml of solution B (approximately 0·2—0·4 μmol of the enzyme) were obtained. These solutions were kept frozen at −20°C and were designated with respect to their pH-optimums (Fig. 2) as "concentrates of enzyme A_8 or B_6".**

Methods

The determination of the dependence of proteolytic activity on pH was effected by the modified method of Anson (with Hb in Britton–Robinson buffers) as described earlier[1,5]. For the determination of proteolytic activity of the fractions, the Britton–Robinson buffer was replaced by Tris-HCl buffer at pH 7. The concentration of the enzyme in the solution was calculated from the extinction coefficient $E^{1\%}_{1cm}$ (pH 5) = 9·04 at 280 nm (*cf.*[1]). The conductivity of the solutions was measured in the Philips Model PE 9 500 apparatus.

The heating of the enzyme solution with or without additive reagents was carried out in the absence of buffers at approximately pH 6·5. Aliquots were removed at intervals and immediately cooled. The chromatography of the thermally treated enzyme was carried out at +3 to +4°C on a standard column of DEAE-Sephadex (1 . 20 cm), equilibrated with 0·01M phosphate at pH 5·9 which was also used as the eluting buffer. (Flow rate 1·5 ml per h). Absorbance was measured at 280 nm. Aliquots (100 μl) were removed from each 1·5 ml fraction and their proteolytic activity (with Hb-substrate, Tris-HCl buffer at pH 7) was determined.

Disc electrophoresis was performed in β-alanine buffer at pH 4·5 (ref.[12]). The N-terminal amino acids were determined by a modification[13,14] of the dimethylaminonaphthalenesulfonation technique after inactivation of the cold enzyme solution by acidification to pH 2 and standing at room temperature for 15 min. The specificity of the isolated forms of the enzymes was examined by digestion of the B-chain of oxidized insulin at 37°C for 2 h. The digests were analyzed by the method of peptide maps as described before[7].

* Abbreviations used: DIP diisopropylphosphoryl, EAC ε-aminocaproic acid, EDTA ethylenediaminetetraacetic acid, Hb hemoglobin, IP-inert protein, TCA trichloroacetic acid, Tris tris(hydroxymethyl)aminomethane, U-urea.

** The pH-optimums of the fractions obtained are indicated by an index at the designation of the fraction throughout the text.

RESULTS

Characterization of Enzyme A and B

The results of disc electrophoresis, amino-acid analysis, and N-terminal end-group analysis of fraction A showed the homogeneity of the preparation and were identical with the results obtained earlier[1]. Fraction B had the same amino-acid composition and the same N-terminal end group yet it gave two zones on disc electrophoresis; these zones, located close to each other, were shifted with respect to the only zone which gave fraction A and their relative intensity varied with each individual preparation. The pH optimum of fraction A (Fig. 2) was 8–9 and that of fraction B was 6. These fractions have been therefore designated A_8 and B_6. The addition of $CaCl_2$ to the B_6-enzyme solution before testing increased its activity whereas its pH-optimum remained unchanged; the activity of fraction B_6 was independent of Ca^{2+}-ions.

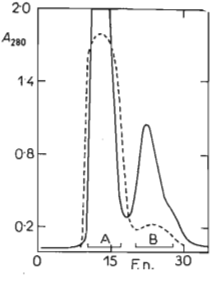

FIG. 1

Chromatography of Desorbed Product on DEAE-Sephadex

The solution (7 ml) of the desalted desorbed product was chromatographed on a 4 . . 65 cm column. Elution by 0·01M phosphate buffer at pH 5·9, fractions 10 ml/20 min, full line — absorbance at 280 nm, dashed line — proteolytic activity (at pH 7) in arbitrary units. A, B, pooled fractions.

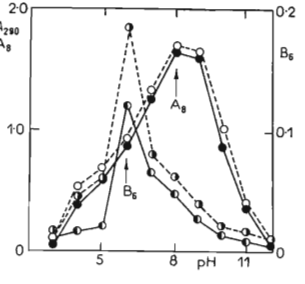

FIG. 2

Dependence of Activity of Original Enzyme on pH

Aliquots corresponding to 5 µl of concentrate of original enzyme A and to 10 µl of original enzyme B were mixed with an equal volume of water or of 0·05M-$CaCl_2$, and used for the digestion of Hb. Full line — activity assayed with Hb as substrate, dashed line — activity in the presence of Ca^{2+}-ions.

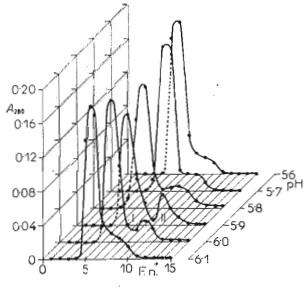

FIG. 3

Effect of pH on Chromatographic Behavior of Thermally-treated Enzyme A_8 on Standard DEAE-Sephadex Column (1 . 20 cm) in 0·01M Phosphate Buffers

I and II are forms of enzymes arisen from original enzyme A_8 by heating. The curves indicate absorbance values of the protein solution after chromatography, *n* fraction number.

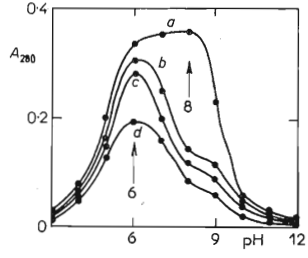

FIG. 5

Changes in pH-Optimums of Pooled Fractions Containing Forms I and Obtained by Chromatography of Enzyme Treated with EDTA

Identical aliquots removed from pooled fractions (Fig. 4) were compared. *a*—*d* products of heating for 0—40 min.

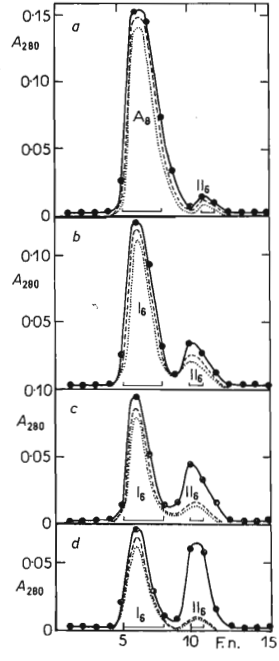

FIG. 4

Effect of EDTA on Transitions of Enzyme A_8 at Increased Temperature as Examined by Chromatography on Standard DEAE-Sephadex Column

a Unheated sample, *b* sample heated at 45°C for 10 min, *c* 20 min, *d* 40 min. A_8 original enzyme; I, II newly formed chromatographically separable forms of enzyme. Full line — absorbance at 280 nm, dotted line — activity assayed with Hb at pH 7 in the absence of Ca^{2+}-ions, dashed line — activity in the presence of Ca^{2+}-ions of 0·01M.

Effect of pH on Chromatography of Separable Forms Obtained by Thermal Treatment of the Enzyme

The concentrate (300 μl) of original enzyme A_8 was diluted by 300 μl of water and the mixture was heated 20 min at 45°C. In a series of experiments, 60 μl aliquots of this mixture were applied to the standard DEAE-Sephadex column, equilibrated with 0·01M phosphate buffer at pH 5·6, 5·7, 5·8, 5·9, 6·0, and 6·1. The chromatographic resolution (Fig. 3) of the two forms of the enzyme (I and II), resulting from the thermal treatment, is very sensitive to pH. Therefore all following separations on the standard DEAE-Sephadex column were carried out at the optimal pH 5·9.

Transition of Enzyme A_8 to form I_6, its Conversion Into Form II_6, and the Overall Denaturation of the Enzyme by Heating with EDTA

The concentrate (160 μl) of original enzyme A_8 was diluted by equal volume of 0·02M solution of EDTA and the mixture was heated at 45°C. Aliquots (40 μl) were withdrawn at intervals of 10, 20, and 40 min. The first aliquot was not heated at all. The aliquots were first cooled, then mixed with 60 μl of water, and the solution chromatographed on the standard DEAE-Sephadex column. The results of these experiments are shown in Fig. 4. The addition of EDTA without heating has no marked effect. With the increasing length of the heating period, however, the peak of enzyme A_8 quickly decreases, peak II on the contrary increases. On prolonged heating, however, only the quantity of protein in peak II increases while the activity rapidly decreases. Peak II thus represents a mixture of the active enzyme and the inert protein, a phenomenon which is in accordance with the existence of two zones observed on disc electrophoresis. The addition of Ca^{2+}-ions (during the activity measurement, not during the heating) obviously has no substantial effect on forms I and II of the enzyme thus formed. As shown in Fig. 5, already after 10 min of heating with EDTA the original form of enzyme A_8 is converted into form I with a pH-optimum at pH 6. This form (I_6), which did not separate chromatographically from the original form A_8, remained even after prolonged heating intact and only its activity decreased in proportion to the decrease of whole peak I_6. By contrast, the pH-optimum of peak II (Fig. 4) remained at the value of 6 corresponding to the unheated sample, regardless of the heating. We designate this form II_6.

Stabilization of Enzyme by Heating with Calcium Ions or with ε-Aminocaproic Acid

The concentrate (160 μl) of original enzyme A_8 was heated to 45°C with the same volume of 0·02M-$CaCl_2$ or 0·02M-EAC solution and then treated in analogy to the preceding experiment in which the effect of EDTA was examined. The chromatographic pattern obtained with the heated and unheated sample on the standard

DEAE-Sephadex column was very similar and indicated only minor changes caused by the heating as far as the transition of forms I to forms II is concerned. This chromatographic pattern can thus be expressed explicitly by the ratio of the area under the peak of form II to the area under the peak of form I. These ratios for the whole series are summarized in Fig. 6 and compared with the same ratios characterizing the preceding experiment.

The pH-optimums of the cleavage of Hb by the pooled fractions derived from the individual peaks were determined in analogy to the preceding experiment. The addition of the stabilizer to original enzymes A_8 and B_6 was without effect on the pH-optimum when the samples were not heated. However, already after 10 min of heating with both types of stabilizers the pH-optimums of forms I were shifted to pH 7. Form I_7 was stable since prolonged heating resulted merely in a slight decrease of its activity leaving the pH-optimum unaltered. The course of the experiment was similar with both types of stabilizers. Forms II of both the heated and the unheated enzyme showed in both cases a stable pH-optimum equaling 6. We designate these forms therefore II_6. Unlike in the preceding case, no marked transition to the inert protein was observed.

Transition of Forms A_8 and B_6 of the Proteinase Caused by 8M *Urea*

Stock solutions were prepared by dilution of the concentrates of the original enzymes by 0·05M ammonium acetate at pH 7 (30 µl of enzyme A_8 + 7·5 ml of buffer, 250 µl of enzyme B_6 + 4·75 ml of buffer). The stock solution (1·1 ml) was diluted by 2·2 ml of water (blank) or by 2·2 ml of 12M urea, either as such or containing $CaCl_2$, EDTA, or EAC (concentration 0·02M). The resulting solution was thermostated at 25°C for 20 min and then 300 µl aliquots were withdrawn for the determination of activity.

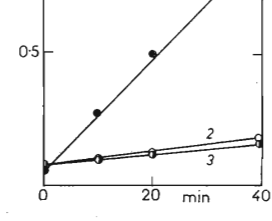

FIG. 6

Ratios of Areas of Peaks II/I in Chromatograms of Enzymes Treated by Different Reagents as Function of Time of Heating

Ordinate: ratio of areas under the peaks II/I, abscissa: time of heating. The enzyme was heated at 45°C. *1* in the presence of 0·01M-EDTA (Fig. 4), *2* of 0·01M-$CaCl_2$, *3* of 0·01M-EAC and chromatographed on the standard DEAE-Sephadex column.

As obvious from Fig. 7, the treatment of the original form A_8 by urea alone markedly decreases its activity and changes its pH-optimum (from 8 to 6). The decrease of the activity of form I_6 is even deeper in the presence of EDTA. By contrast, the presence of Ca^{2+}-ions and EAC in urea slightly increases the activity of the enzyme leaving the pH-optimum unaltered (I_8). The effect of urea on the original form of enzyme B_6 is reversed. The activity increases markedly in all cases and the pH-optimum is changed by treatment with urea alone from 6 to 8 (form II_8 appears) whereas Ca^{2+}-ions and EAC when present in the urea solution lead merely to a shift of the optimum to pH 7 (form II_7). The curve characterizing the sample treated with EDTA in urea shows a flat maximum ranging from pH 7 to pH 8.

Chromatography of Urea-treated Enzyme A_8 on DEAE-Sephadex

The concentrate (0·3 ml) of the original enzyme A_8 was incubated with 0·6 ml of 12M urea 20 min at 25°C. The solution was then freed of urea on a 1·2 . 54 cm column of Sephadex G-25 in 0·05M ammonium acetate at pH 7 and +4°C (fractions 3 ml : : 10 min). Pooled fractions No 6–8 containing the proteinase were treated with acetone to precipitate the proteinase and 0·6 ml of the concentrate was obtained. The latter was chromatographed on the standard DEAE-Sephadex column (Fig. 8). The dependence of proteolytic activity on pH of the pooled fractions derived from

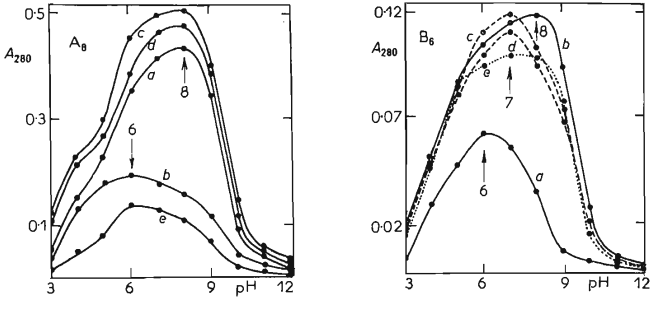

FIG. 7

Transitions of pH-Profiles of Original Forms of Enzyme A_8 and B_6, Brought about by Treatment with 8M Urea at 25°C in Presence of 0·013M Concentration of Additive Reagents or in their Absence

Treatment in the absence of urea (*a*), in the presence of urea (*b*), urea and Ca^{2+}-ions (*c*), urea and EAC (*d*), urea and EDTA (*e*).

the two peaks was examined (aliquots 100 µl, Fig. 9). The part of pooled fraction derived from peak II was examined once more after the incubation of an 0·6 ml aliquot with 0·6 ml of 0·02M-EDTA, 10 min at 45°C (in the absence of urea). Aliquots 200 µl in volume were withdrawn for the activity test (Fig. 9). As can be seen from the Figure, the isolated form I of the enzyme shows – like in the experiment documented by Fig. 7 – a pH-optimum of 6 whereas the isolated form II has its pH-optimum at 7 (unlike the pH-optimum of 8, Fig. 7). This pH-optimum, however, is reversed to pH 6 and the activity is lower after the heating with EDTA. When the part of the solution of form II_7 was incubated 10 min at 45°C only with water not containing EDTA, the enzyme had the same properties as the unincubated sample.

Effect of Urea Alone or in the Presence of Ca^{2+}*-Ions on Forms* I_6 *and* II_6 *of Enzymes Prepared by Heating of Original Enzyme* A_8 *with EDTA*

The concentrate (1·4 ml) of original enzyme A_8 was heated with 1·4 ml of 0·02M-EDTA 20 min at 45°C and after cooling chromatographed on the standard DEAE-Sephadex column. The fractions derived from peak I_6 and II_6 were pooled (final volumes 70 ml). An 0·6 ml sample was always mixed with 1·2 ml of 12M urea alone or containing 0·02M Ca^{2+}-ions and kept 20 min at 25°C. The pH-optimum was determined afterwards. The pH-optimum of form I_6 remained at the original value of 6, its activity, however, was slightly increased after treatment with urea alone, markedly after treatment with urea and Ca^{2+}-ions. The activity of form II_6 also increased slightly after the treatment with urea and its pH-optimum became broader and ranged from 6 to 7; in the presence of Ca^{2+}-ions in urea the activity markedly increased and the pH-optimum was shifted to 7.

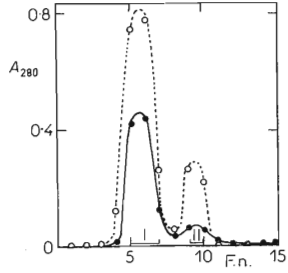

FIG. 8

Chromatography of Urea-Treated Original Enzyme A_8 on Standard DEAE-Sephadex Column

I, II forms of the proteinase separable by chromatography. Full line – absorbance at 280 nm, dashed line – activity assayed with Hb at pH 7.

Specificity of Cleavage of B-*Chain of Oxidized Insulin by Different Forms of Enzyme*

The following 6 samples gave identical peptide maps represented in Fig. 10*a*:

Type of preparation of sample before chromatography on DEAE Sephadex	Form
1 Concentrate of original enzyme	A_8
2 Product of heating of A_8 with EDTA	I_6
3 Product of heating of A_8 with EDTA	II_6
4 Product of dialysis of A_8 against water	I_7
5 Product of dialysis of A_8 against water	II_6
6 Product of urea treatment of A_8	I_6
7 Product of urea treatment of A_8	II_7

Only the seventh sample differed from the others and it is represented by the peptide map given in Fig. 10*b*. This last sample yielded a digest whose map in repeated experiments contained one additional spot. The peptide giving this spot was isolated from the map, subjected to amino-acid analysis and found to represent the N-terminal pentadecapeptide of the B-chain:

1.				5.					10.					15.
(Phe,	Val,	Asn,	Gln,	His,	Leu.	$CySO_3H$,	Gly,	Ser,	His,	Leu,	Val,	Gln,	Ala,	Leu)
0.6	1.9	0.9	1.9	1.6	2.7	0.9		1.2	0.8				1.0	

For the calculation of the molar ratios, the value of alanine (in μmol) was taken to represent one residue. The value of N-terminal phenylalanine is lower due to its reaction with ninhydrin.

DISCUSSION

Sipos and Merkel[15,16] have shown in their interesting studies that the heating of the proteinase from a marine bacterium, of trypsin and chymotrypsin in the presence

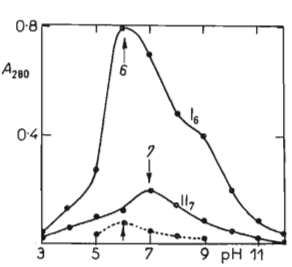

FIG. 9

Dependence of Activity on pH of Both Forms of Enzyme, I and II, Prepared by Chromatography of Urea-Treated Enzyme A_8 as Illustrated by Fig. 8 (Full line)

Dotted line pH-optimum of the equivalent part of solution corresponding to peak II, incubated 10 min at 45°C in the presence of 0·01M-EDTA (in the absence of urea).

of Ca^{2+}-ions leads to a conformational change of these proteins which manifests itself by a higher stability and by shift of the temperature optimum. The degree of the proteinase and esterase activity of these enzymes was unevenly affected.

We have shown that not only the thermal treatment but also the treatment with urea – *i.e.* a treatment carried out under conditions generally near to those of the transition state – can be the cause of such changes of the proteinase which may

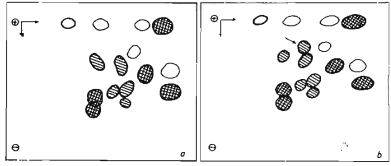

FIG. 10

Peptide Maps of B-Chain of Oxidized Insulin Digested by Different Forms of Enzyme, Arison by Treatment of Alkaline Proteinase from *A. flavus* with Urea

Vertically: electrophoresis, pH 1·9, horizontally: chromatography in the system butanol–pyridine–acetic acid–water (45 : 30 : 9 : 36). *a* form I_6, *b* form II_7. The arrows point to the differing peptide.

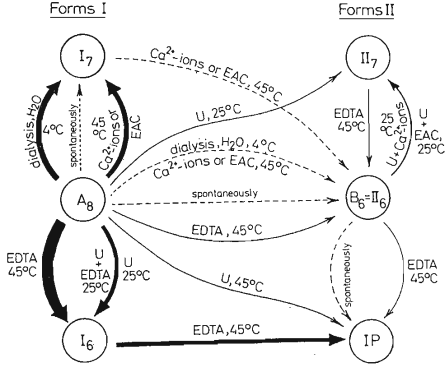

FIG. 11

Scheme of Transition of Forms of Alkaline Proteinase from *A. flavus*

The thickness of the arrow indicates the rate of the transition. Slow transitions are marked by dashed lines. Forms A_8 and I_6 do not change their pH-optimums after treament with urea and Ca^{2+}-ions or with urea and EAC (25°C), they increase, however, their activity. Form I_7 is relatively the most stable one, form II_7 shows a different specificity.

affect not merely its stability but also its pH-profile and also its proteolytic specificity. Inside this sensitive range an important role play besides Ca^{2+}-ions also EAC and EDTA.

The results obtained by us so far are shown schematically in Fig. 11. Alkaline proteinase A_8, which shows a pH-optimum between 8 and 9 when assayed with hemoglobin as substrate (Fig. 1 and 2), is most probably the form of the enzyme secreted by the mold *Aspergillus flavus*. In the process of its isolation a small part of the enzyme undergoes spontaneous conversion into form B_6 and into inert protein IP. The enzyme is converted spontaneously also into form I_7; this conversion depends on the ionic strength[5], higher values stabilize form A_8. When we removed the inorganic salts from the starting crude preparation[1] by dialysis against water or by chromatography on Sephadex in water, form A_8 disappeared completely and was converted for the most part into form I_7. Form A_8, however, remained unchanged after dialysis or chromatography on Sephadex carried out with buffers in the cold. Form II_6 can be regarded as identical with form B_6 of the original preparation.

Ca^{2+}-ions and EAC showed the like effect which was marked even at relatively low concentrations. The effect of EAC deserves special interest since unlike Ca^{2+}-ions – the known activator or stabilizer of a number of enzymes – it is used at high concentrations as an inhibitor of proteolysis[17–19]. EDTA at the same concentration showed an effect opposite to Ca^{2+}-ions and EAC. The necessary condition under which these additive reagents were effective was increased temperature, close to the transition temperature. The alkaline proteinase from *A. flavus* loses activity[5] at approximately 50°C. The temperature of 45°C was found to be the most convenient for these conversions and the enzyme was very sensitive to the reagents at this temperature. In parallel experiments carried out in the cold (+4°C) we did not detect any effect of the additive reagents or any transitions of the forms as long as the ionic strength did not decrease. (The concentration of 0·05M ammonium acetate (pH 7) was sufficient to stabilize the enzyme in the cold.) At increased temperatures not exceeding 45°C (*i.e.* at 40°C, 35°C) similar, yet less marked effects were observed after longer periods of treatment with additive reagents. Minor changes occur even at room temperature round 25°C. It is, therefore, absolutely necessary for the preparation of the enzyme to handle the supplied culture medium and the subsequent products of the isolation procedure in the cold at +4°C. At this low temperature, *e.g.* form A_8 dissolved in the EDTA solution cannot be affected even by repeated passage through a column of Sephadex G-25 equilibrated with 0·02M-EDTA. The enzyme eluted from this column and then separated from EDTA on another Sephadex G-25 column (containing 0·05M ammonium acetate at pH 7) showed the same pH-profile of activity with a maximum between 8 and 9 as the enzyme used for the experiment. Hence, at the low temperature the ionic strength of the EDTA solution played the decisive role and not its ability to bind metal ions in a complex. EDTA acted therefore in the cold as a stabilizing factor.

The conversions could be brought about besides heating also by treatment with urea. We made use of the fact that 8M urea – unlike 6M guanidine hydrochloride – does not denature irreversibly our enzyme[5]. The conversions could be brought about at room temperature (25°C). At 45°C the alkaline proteinase is inactivated by 8M urea.

Another necessary condition of the conversion of forms I into forms II – in addition to higher temperature or presence of urea – is the enzymatic activity itself. We tried to heat the enzyme A_8 in the form of its DIP-derivative. As we showed by chromatographic experiments, no conversion into form II occurred even after 40 min of heating at 45°C. The pathways of conversions shown schematically in Fig. 11 are those which we considered proved in independent experiments. We do not consider here, *e.g.* form II_8 which we have not been able to isolate on a preparative scale (Fig. 8, 9), even though the experiments with the addition of urea to the solution of enzyme B_6 (Fig. 7) seemed to indicate its existence. The reversibility of the conversion of forms II into forms I has not been observed.

The presented series of experiments shows that the alkaline proteinase from *A. flavus* can exist in at least five enzymatically active forms which significantly differ from each other in their pH-activity profile when assayed with hemoglobin as substrate and/or in chromatographic and electrophoretic characteristics; they also differ in their ability to undergo further transitions. Attempts to separate form A_8, I_7, and I_6 chromatographically or electrophoretically were unsuccessful; we designate them "forms I". A similar group called "forms II" represent $B_6 = II_6$, II_7, an IP. The inactive inert protein differs little yet distinctly from II_6 on disc electrophoresis; it is contained in peak II and sometimes when chromatographed it is a little shifted with respect to form II_6 (which it overlaps for the most part) toward higher elution volumes. (It is likely that conditions of its complete separation can be found). Form II_7 differs from the remaining forms in addition to the pH-profile also in its specificity of cleavage of the B-chain of oxidized insulin. Form II_7 liberates at a high concentration the uncleaved N-terminal pentadecapeptide which has not yet been found in digests of insulin effected by other preparations of alkaline proteinase[3].

For the ability of the alkaline proteinase to undergo conversions into a number of different forms we propose the term polymorphism. This phenomenon may be of biological importance since it extends the action of the extracellular proteinase in the medium in which the mold grows. It may bear relation to the fact that our proteinase – similarly to many other extracellular microbial enzymes – is lacking disulfide bonds[1] and the conversions can be thus easier.

At the present stage of our research we can prove merely the existence of the polymorphism of the alkaline proteinase from *A. flavus* and to propose the lines of approach leading to the preparation of the individual forms. We have adopted the working hypothesis of individual forms being conformers specifically affected by ionic

interactions. A more detailed characterization of the individual forms would require – besides others – activity tests with a number of other substrates and especially a series of material-consuming physico-chemical studies. The described conversions may prove useful, however, already at present in studies on the polymorphism of other enzymes.

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