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Proteinase from germinating bean cotyledons

Evidence for involvement of a thiol group in catalysis

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To degrade storage proteins germinating seeds synthesize proteinases *de novo* that can be inhibited by thiol-blocking reagents [Baumgartner & Chrispeels (1977) *Eur. J. Biochem.* **77**, 223–233]. We have elaborated a procedure for isolation of such a proteinase from the cotyledons of *Phaseolus vulgaris*. The purification procedure involved fractionation of the cotyledon homogenate with acetone and with $(NH_4)_2SO_4$ and successive chromatographies on DEAE-cellulose, activated thiol–Sepharose and Sephacryl S-200. The purified enzyme has an M_r of 23400, proved to be highly specific for the asparagine side chain and blocking of its thiol group resulted in loss of the catalytic activity. The chemical properties of the thiol group of the bean enzyme were investigated by acylation with t-butyloxycarbonyl-L-asparagine *p*-nitrophenyl ester and by alkylations with iodoacetamide and iodoacetate. Deviations from normal pH–rate profile were observed, which indicated that the thiol group is not a simple functional group, but constitutes a part of an interactive system at the active site. The pK_a value for acylation and the magnitude of the rate constant for alkylation with iodoacetate revealed that the bean proteinase possesses some properties not shared by papain and the other cysteine proteinases studied to date.

Proteolytic enzymes play a central role in the biochemical mechanism of germination. Their main function is the hydrolysis of the stored proteins of the seed, which ensures protein synthesis for the developing plant (Ashton, 1976). Germination of leguminous seeds is accompanied by a dramatic increase in proteolytic activity in the cotyledons owing to synthesis of proteolytic enzymes *de novo*. Baumgartner & Chrispeels (1977) reported the purification and characterization of such an enzyme, the major endopeptidase of mungbean (*Vigna radiata*) cotyledons. They suggested that the enzyme belongs to the class of cysteine proteinases, as indicated by its activation by thiol-containing compounds and by its inactivation by thiol-blocking reagents. Proteinases of other germinating seeds could also be activated by thiol-containing molecules (Sundblom & Mikola, 1972; Yomo & Varner, 1973; Basha & Beevers, 1975). However, it should be kept in mind that such activation and inactivation studies do not prove the direct involvement of the thiol group in the catalysis. In fact, in some serine proteinases, e.g. in carboxypeptidase Y (Bai & Hayashi, 1979), a thiol group resides near the active site so that its chemical modification can lead to inactivation of the enzyme.

The aim of the present paper has been (1) to obtain more direct evidence with respect to the participitation in the catalysis of the thiol group of a leguminous seed proteinase, and (2) to delineate any mechanistic relationship between the seed proteinase and papain, which has long been regarded as archetype of cysteine proteinases (for a review see Polgár & Halász, 1982). To this end we elaborated a simple method for isolation of the cysteine proteinase synthesized during germination of bean seeds and examined the chemical reactivity of its thiol group. It was found that, though the bean proteinase resembled papain in some respects, it exhibited considerable differences as well.

Abbreviations used: Boc-, t-butyloxycarbonyl-; Z-, benzyloxycarbonyl-; ONp, *p*-nitrophenyl ester.

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Materials and methods

Materials

Activated thiol–Sepharose 4B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals. Aldrithiol-2 (2,2′-dipyridyl disulphide) was the product of Aldrich–Europe. Boc-Asn-ONp, Z-Gly-ONp, Z-Ala-ONp and Z-Tyr-ONp were obtained from Sigma Chemical Co., and Boc-Gln-ONp was purchased from Serva. All other reagents were of analytical grade.

Enzyme assay

Azocasein substrate, which was prepared as described by Hazen *et al.* (1965), was used to monitor enzymic activity during preparation. The assay mixture contained 1.0ml of proteinase sample, 4.0ml of 0.1M-sodium phosphate buffer, pH6.5, containing 1mM-EDTA and 1mM-cysteine, and 5.0ml of 2% (w/v) azocasein solution adjusted to pH6.5. This reaction mixture was incubated at 25°C. Every 3min a 1.0ml sample taken from the reaction mixture was added to 1.0ml of 15% (w/v) trichloroacetic acid. The precipitates formed after standing for 15min at room temperature were centrifuged down, and the supernatants were combined with equal volumes of 1M-NaOH (1ml each). The absorbances of the resulting yellow solutions were measured at 436nm, and the values were plotted against time. From the linear part of the curve the absorbance change per 10min was calculated. One unit of enzyme activity represents 1 unit of absorbance change under the above conditions.

Enzyme purification

Seeds of beans (*Phaseolus vulgaris* 'Juliska', 'Cherokee' or unknown varieties) were obtained from local dealers and were germinated for 5 days at 20°C as described by Yomo & Srinivasan (1973). Then the cotyledons (400g) were homogenized in a Waring blender (2min at full speed) at room temperature in 25mM-sodium citrate buffer, pH5.7, containing 5mM cysteine, 2mM-EDTA and 0.02% NaN_3 (600ml, 4°C). The homogenate was centrifuged at 5°C in a Beckman J21B centrifuge at 10000*g* for 15min (or in a Sorvall RC-3B centrifuge at 6500*g* for 30min), and the supernatant was collected. The sediment was resuspended in 300ml of the homogenization buffer, centrifuged again at 10000*g* for 15min (or 6500*g* for 30min), and the second supernatant was combined with the first one.

Acetone precipitation. The cold supernatant (900ml) was saturated at room temperature up to 15% with acetone (158ml) previously equilibrated to −5°C. The acetone was slowly delivered (10min) into the stirred solution through a capillary tube. The mixture was immediately centrifuged as above. The sediment was discarded, and the supernatant (930ml) was used for the next purification step.

$(NH_4)_2SO_4$ precipitation. The cysteine proteinase was precipitated from the acetone/buffer mixture with $(NH_4)_2SO_4$ (51g added to 100ml of solution). Possible change in pH was not adjusted. The salt was added during 30min with stirring at 0°C. Then the mixture was centrifuged, which separated it into three phases, so that the solid precipitate was positioned between the lower salt solution and the upper acetone layer. The two liquid phases could be removed simply by pouring out the solution from the tube. The solid cake remaining in the tube was mixed in the Waring blender with 200ml of cold 25mM-sodium citrate/phosphate buffer, pH5.7, containing 5mM-cysteine, 2mM-EDTA and 0.02% NaN_3, centrifuged and the sediment extracted with 100ml of the same buffer. The supernatants were combined (360ml).

Dialysis. The supernatant was dialysed in the cold-room for 24h against 2×10 litres of 5mM-cysteine containing 1mM-EDTA, pH7.0 (change after 16h).

DEAE-cellulose chromatography. After dialysis the enzyme solution was centrifuged at 10000*g* for 15min at 5°C, and to the clear supernatant (470ml) was added Whatman DE-23 DEAE-cellulose (80ml) that had been equilibrated with 20mM-sodium phosphate buffer, pH6.5, containing 1mM-EDTA (equilibration buffer). The suspension was slowly stirred for 30min and stored overnight in the cold-room. Then the cellulose was filtered on a Buchner funnel at room temperature, washed with 160ml of equilibration buffer and, after resuspension in the same buffer, the cellulose was filled into a chromatographic column (4.2cm×6cm). The column was washed in the cold-room with the equilibration buffer containing 0.1M-NaCl (240ml/h) until the absorption at 280nm decreased to almost zero. The enzyme was eluted with the equilibration buffer containing 0.5M-NaCl. The fractions containing the single protein peak were collected (270ml).

Covalent chromatography. The cysteine proteinase obtained from the DEAE-cellulose was precipitated by slowly adding solid $(NH_4)_2SO_4$ (about 30min) up to 80% (56.1g added to 100ml of solution). After keeping in the refrigerator for 30min the precipitate was collected by centrifugation at 10000*g* for 15min. The sediment was dissolved in 20ml of equilibration buffer, and after centrifugation the supernatant (about 25ml) was loaded on to an activated thiol–Sepharose 4B column (2.4cm×5.5cm) that had previously been washed with equilibration buffer containing 0.3M-NaCl. The flow rate was 40ml/h throughout the

chromatography. The column was washed with the same buffer until the absorption at 280nm decreased to zero. The thiol-containing proteins were eluted with the equilibration buffer containing 10mM-cysteine. Fractions of volume 2–3ml were collected and their activities were measured with azocasein or more conveniently with Boc-L-asparagine *p*-nitrophenyl ester substrate. In the latter case the effect of cysteine on substrate hydrolysis was taken into account. The active fractions were combined (25–30ml) and freeze-dried. The column was regenerated by washing successively with (1) 6M-urea containing 10mM-cysteine (100ml), (2) equilibration buffer containing 0.3M-NaCl, (3) 1mM-2,2'-dipyridyl disulphide in 20mM-sodium phosphate buffer, pH8.0, and finally (4) equilibration buffer containing 0.3M-NaCl.

Gel chromatography. The freeze-dried proteinase was dissolved in 1ml of distilled water, the insoluble material was removed by centrifugation, and the clear supernatant was applied to a Sephacryl S-200 column (0.6cm × 128cm) previously washed with 20mM-sodium phosphate buffer, pH6.5, containing 1mM-EDTA and 5mM-cysteine. Fractions of volume 1.25ml were collected at a flow rate of 2ml/h, and those containing the enzymic activity were combined (3.6ml) and freeze-dried.

Polyacrylamide-gel electrophoresis

Disc gel electrophoresis was carried out under non-denaturing conditions in 7.5% polyacrylamide gels with Tris buffer, pH8.9 (Davis, 1964). The electrophoresis was run in the refrigerator at 2mA/tube for the first 20min and then at 4mA/tube until the tracking dye (Bromophenol Blue) reached the end of the gels (about 2h).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in a vertical-slab-gel apparatus utilizing a pore-size-gradient system (4–28% polyacrylamide). The electrophoresis was run overnight at room temperature.

Determination of M_r

The M_r was measured by the above dodecyl sulphate/polyacrylamide-gel electrophoresis or by gel filtration on a Sephadex G-75 column (0.6cm × 60cm) equilibrated with homogenization buffer. Marker proteins used for calibration were bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), chymotrypsinogen (M_r 25000), soya-bean trypsin inhibitor (M_r 21500) and ribonuclease (M_r 13800).

Kinetic measurements

Enzyme activities with synthetic substrates. Enzyme assays with Boc-aminoacyl-ONp and Z-aminoacyl-ONp substrates were performed in 0.1M-sodium phosphate buffer, pH6.5, containing 1mM-EDTA at 25°C. To 1ml of buffer 20μl of substrate (1mg/ml in acetonitrile) was added together with 50μl of proteinase (0.15 unit/ml). The liberation of nitrophenol was recorded at 348nm by using the 0–0.1 absorbance range of a Cary 118C or a Varian 634 spectrophotometer. The measured rates were corrected for non-enzymic spontaneous degradation of the substrate.

Acylation reaction. Acylation of the bean proteinase was measured in 0.1M-sodium acetate, -sodium citrate or -sodium phosphate buffer with Boc-Asn-ONp (3.7μM) under pseudo-first-order conditions. The reaction was measured in a semi-micro cell, so that 50μl of enzyme (0.15 unit/ml) was added to 1.0ml of reaction mixture. Liberation of *p*-nitrophenol and *p*-nitrophenolate was monitored at 348nm by using the 0–0.05 absorbance range of the spectrophotometer at 25°C. Spontaneous hydrolysis of the substrate at high pH was taken into account.

Alkylation reactions. Alkylations with iodoacetamide and iodoacetate were measured under pseudo-first-order conditions, the alkylating agents being in excess. The reactions were carried out at 25°C in 0.1M-sodium acetate, -sodium citrate or -sodium phosphate buffer. In order to rule out any special buffer effects, an overlap between the different buffers was ensured. Samples of the reaction mixture were withdrawn at appropriate times and their enzymic activity was determined with Boc-Asn-ONp substrate. The second-order rate constants were calculated by dividing the first-order rate constants by the concentration of the alkylating agent used.

Results and discussion

Enzyme purification

In agreement with previous studies (Yomo & Srinivasan, 1973; Chrispeels & Boulter, 1975), we found that after a lag period of about 2 days the proteolytic activity of bean cotyledons begins to increase and reaches a maximum after about 5 days' germination. At this stage more than 90% of the caseolytic activity could be inhibited with *p*-chloromercuribenzoate.

For preparation of mung-bean proteinase, Baumgartner & Chrispeels (1977) employed a chromatographic column containing crude proteinase inhibitors covalently bound to Sepharose. Using a similar column in the preparation of the proteinase from *Phaseolus vulgaris*, we lost a considerable amount of proteinase activity. Additionally, the extract gave an opalescent supernatant that was unsuitable for application to the chromatographic column. Instead, we treated the solution with acetone. This resulted in an abso-

lutely clear supernatant, from which the enzyme was precipitated with $(NH_4)_2SO_4$. After the protein had been dissolved and the solution dialysed, the enzyme was adsorbed on DEAE-cellulose. Because of the relatively high viscosity of the solution, this process was carried out by the batch technique. Some active enzyme remained in solution at this step. Nevertheless, the specific activity of the cystein proteinase increased by about an order of magnitude (Table 1).

Another substantial enhancement in specific activity was attained by covalent chromatography on the activated thiol–Sepharose column. The mixed disulphide of the column, which is formed between the glutathione spacer arm and 2-thiolpyridine (i.e. the thiol form of 2-thiopyridone), reacts specifically with thiol-containing proteins (Brocklehurst, 1979). Unfortunately, the yield was not high at this chromatographic step (Table 1). In initial attempts the enzyme was bound to the column immediately after elution from the DEAE-cellulose either by the batch technique or by loading the eluent on to the column. Better results were obtained, however, if the enzyme was applied to the column in a more-concentrated solution after precipitation with $(NH_4)_2SO_4$ and dissolution in a small volume. On washing the column with cysteine, the enzymic activity was eluted in a single peak along with a considerable amount of 2-thiopyridone.

The last step of purification was gel chromatography on Sephacryl S-200 (Fig. 1). Surprisingly, the proteinase emerged from the column in the low-M_r fractions just before cysteine, which together with 2-thiopyridone was present in the material applied to the column. Two active peaks were found when tested with Boc-Asn-ONp (Fig. 1). The first peak was due to the proteinase; the second one did not represent enzymic reaction but was due to the decomposition of the active ester

Table 1. *Purification of bean proteinase*

The purification was from 400g of cotyledons. For experimental details see the Materials and methods section.

Step	Volume (ml)	A_{280}	Caseolytic activity (units/ml)	$10^3 \times$ Specific activity (units/A_{280})	Activity yield (%)
Extract supernatant	900	75.6	0.071	0.94	100
Acetone pptn. supernatant	930	—*	0.048	—*	70
$(NH_4)_2SO_4$ pptn. supernatant	320	—*	0.110	—*	55
After dialysis	470	20.2	0.065	3.21	48
DEAE-cellulose chromatography	270	2.1	0.059	28.1	25
Covalent chromatography	29	—*	0.198	—*	9
Sephacryl S-200 chromatography	3.6	1.3	1.24	956	7

* The values were not determined when acetone or 2-thiopyridone, each having high absorbance at 280nm, was present.

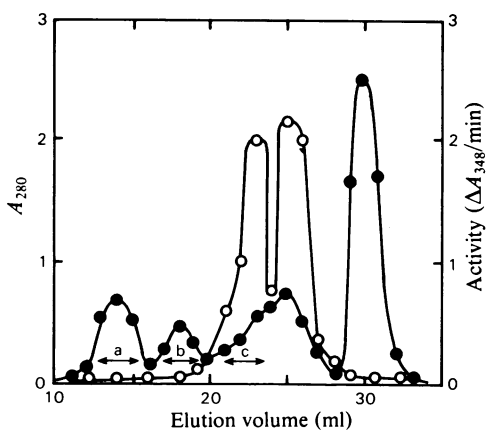

Fig. 1. *Gel chromatography of bean proteinase on Sephacryl S-200*

Chromatographic conditions are described in the Materials and methods section. Protein content (A_{280}, ●) was measured at 280nm. Enzymic activity (ΔA_{348}/min, ○) was determined with Boc-Asn-ONp as substrate. The double arrows indicate the pooled fractions.

substrate by cysteine. Thus, if azocasein was used as substrate, only the first peak was active. Well-separated from cysteine, 2-thiopyridone appeared beyond the column volume. These results indicated that the cysteine proteinase of bean cotyledons and 2-thiopyridone were retarded on Sephacryl S-200, probably as a consequence of their hydrophobic character. Serum albumin, ovalbumin, chymotrypsinogen and trypsin inhibitor were eluted unretarded.

Adsorption phenomena on Sephacryl S-200 have been observed before, but this occurred below pH5.5 and above pH8 rather than at near neutrality (Belew *et al.*, 1978). The considerable degree of purification at the gel-chromatographic step (Fig. 2) may be due to some interaction between the bean proteinase and Sephacryl S-200. The analysis by polyacrylamide-gel electrophoresis (Fig. 2) shows that the proteinase is separated from a very complex protein mixture in almost pure form. A sharp band running along with Bromophenol Blue is also apparent in each gel tube, particularly in the gel containing the purified proteinase (tube c). This may be due to contamination by some low-M_r protein(s) not sieved in 7.5% polyacrylamide gel. The contamination, however, disappears when the bean proteinase is gel-filtered on a Sephadex G-25 column. This procedure was always employed before the enzyme was used for kinetic measurements. Protein contaminations in the purified enzyme were less than 5%, as estimated by comparison of staining of increasing amounts of protein. The extent of enzyme purification is given in Table 1.

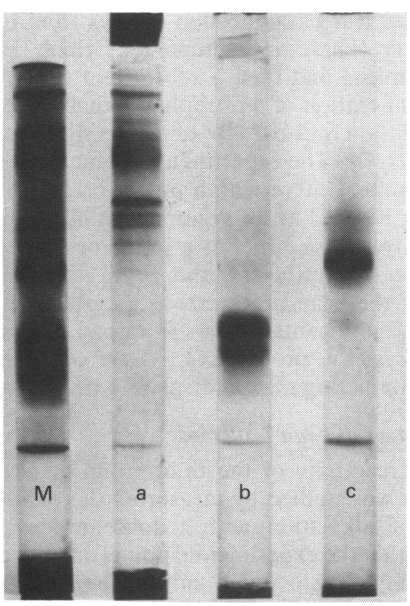

Fig. 2. *Gel-electrophoretic pattern of bean proteinase before and after chromatography on a Sephacryl S-200 column*

M, the protein mixture collected from thiol–Sepharose and applied to Sephacryl S-200; a, b and c are the protein peaks in the order of elution from Sephacryl S-200 (see Fig. 1). Tube c represents the nearly pure proteinase.

Determination of M_r

In contrast with Sephacryl S-200, Sephadex G-75 did not retard the bean proteinase, and gel chromatography on this column yielded an M_r of 23800. In agreement with this result, dodecyl sulphate/polyacrylamide-gel electrophoresis showed an M_r of 23000. These values are similar to the M_r value of 23000 found for the mung-bean proteinase (Baumgartner & Chrispeels, 1977).

Effect of inhibitors on proteinase activity

Di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride are potent inhibitors of serine proteinases, but different enzymes exhibit remarkably different susceptibilities towards the two inhibitors. Therefore, in a search for serine proteinases both inhibitors should be tested. However, the bean enzyme was not inhibited by these compounds (1mM inhibitor, 25°C, 30min preincubation). EDTA, an inhibitor of metalloenzymes, did not show any effect either, so that 1mM-EDTA could be employed during the isolation procedure. On the other hand, the cysteine-enzyme inhibitors *p*-chloromercuribenzoate (0.2mM, 25°C, 1min) and *N*-ethylmaleimide (2mM, 25°C, 30min) completely inhibited the bean proteinase. Iodoacetamide and iodoacetate (see below) displayed a similar effect.

Specificity of bean proteinase

This enzyme is expected to be similar to or identical with the proteinase isolated from mung bean. In order to decide about possible similarity or identity, we compared the substrate specificities of the two species. The specificity of the enzyme was determined by measuring the activity of the purified enzyme towards synthetic substrates used previously for the mung-bean enzyme. Table 2 shows that the asparagine derivative is the best substrate. Surprisingly, the glutamine derivative is a very poor substrate. This is different from the result found with the mung-bean enzyme (Baumgartner & Chrispeels, 1977), where the enzyme hydrolysed the glutamine compound only slightly more slowly than the asparagine derivative, and the reactivities of the glycine and alanine derivatives were similar to each other and in the order of magnitude of the reactivities of the asparagine and glutamine derivatives (cf. Table 2). The dissimilar

Table 2. *Hydrolysis of synthetic substrates by bean proteinase*
Conditions for activity measurements are described in the Materials and methods section.

Substrate	Enzymic activity: With *Phaseolus vulgaris* enzyme [$\Delta A_{348} \cdot min^{-1} \cdot unit^{-1}$]	(%)	With *Vigna radiata* enzyme* (%)
Boc-Asn-ONp	3.18	(100)	100
Boc-Gln-ONp	0.04	(1.3)	73
Z-Gly-ONp	<0.03	(<1)	20
Z-Gly-Pro-ONp	0.09	(3)	
Z-Ala-ONp	0.06	(2)	20

* Baumgartner & Chrispeels (1977).

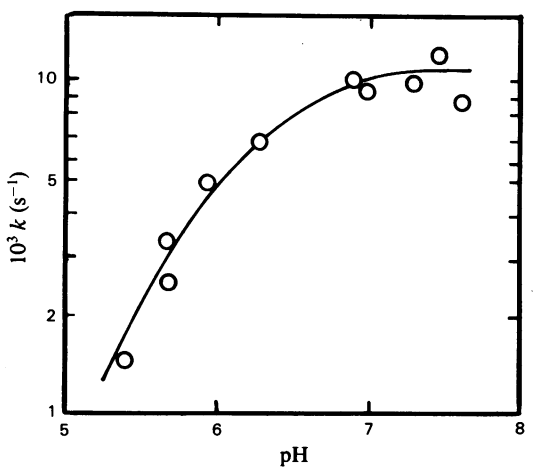

Fig. 3. *pH–rate profile of the reaction of bean proteinase with Boc-Asn-ONp*
The continuous line is the theoretical curve for ionization of a group of $pK_a = 6.15$.

assay conditions used with the mung-bean enzyme (0.1 M-Tris buffer, pH 7.1, 15% methanol) did not affect the relative substrate specificities (results not illustrated). The fact that the bean enzyme failed to hydrolyse Boc-Gln-ONp at an appreciable rate cannot be ascribed to poor quality of the substrate, because (1) after hydrolysis of the compound glutamic acid was the only amino acid that could be found, (2) elementary analysis gave correct values for total nitrogen and amide nitrogen, and (3) contamination by some inhibitory activity of the preparation seemed to be unlikely because addition of the glutamine derivative to the assay mixture for the asparagine derivative did not interfere with the hydrolysis of the latter. Accordingly, the specificity of our enzyme is considerably different from that of the enzyme obtained from mung bean. It may be noted in this respect that acylase II, for example, exhibits much higher specificity towards acyl-aspartates than towards the corresponding acyl-glutamates (Birnbaum, 1955).

pH-dependence of substrate hydrolysis

We measured the pH-dependence characteristic of acylation of the bean proteinase with Boc-Asn-ONp (Fig. 3). The reactions were followed under pseudo-first-order conditions, i.e. below the K_m of substrate, which was independently determined from Lineweaver–Burk plots (21 μM at pH 6.5). To obtain the second-order rate constant of acylation, which also involves binding, the pseudo-first-order rate constant must be divided by the molarity of the enzyme in the reaction mixture (cf. Bender & Kézdy, 1965). Since this was not known, in Fig. 3 the pseudo-first-order rate constants were plotted. The pH-dependence gave the pK_a of the ionizing group that is important in acylation. That acylation is a true catalytic step follows from our preliminary 'burst' measurements, which imply a rapid initial and then a subsequent slow, steady-state, liberation of *p*-nitrophenol that corresponds to acylation and the rate-determining deacylation respectively. The experimental points on Fig. 3 fit a theoretical curve with a pK_a of 6.15. This value cannot be used as an argument in classifying the bean proteinase either as a serine or as a cysteine enzyme. Nevertheless, the result clearly shows that, if the bean proteinase is a cysteine enzyme, then it significantly differs from papain and from other cysteine proteinases, whose cysteine–histidine interacting system displays a pK_a of about 4.

Alkylation of bean proteinase

The reactivity of the thiol group of bean proteinase was studied by measuring the pH-dependence of alkylation with iodoacetamide. Fig. 4 shows that the experimental points do not conform to a simple dissociation curve. The experimental points could not be determined in the alkaline pH range because the enzyme, quite unusually, was slowly inactivated even at pH 7.5 and more rapidly at higher pH values. Thus the pK_a that can be calculated from the theoretical curve in Fig. 4 is a lower limit (7.7), and the true value might be higher, such as that found with papain (8.5; cf. Polgár & Halász, 1982).

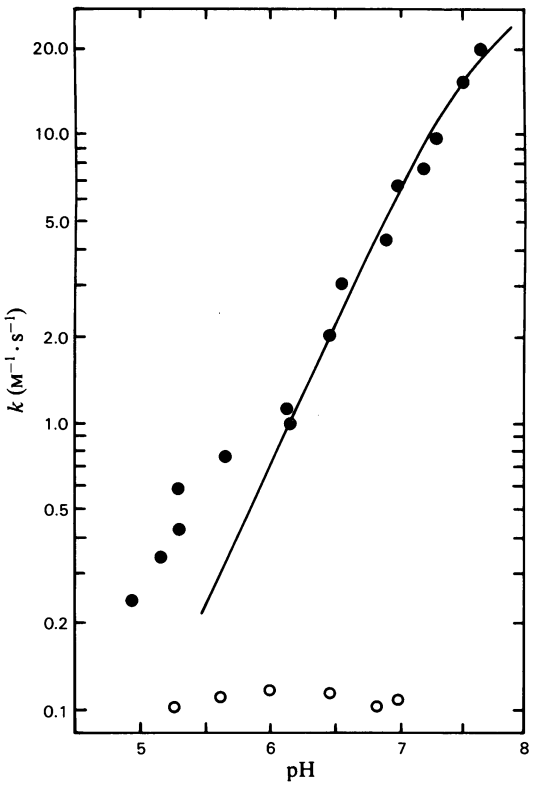

Fig. 4. *pH-dependence of the second-order rate constants of alkylations of bean proteinase*

Alkylations were performed with 0.1–1.0mM-iodoacetamide (●) and 5–10mM-iodoacetate (○). The continuous line is a theoretical curve for the dissociation of an ionizing group with $pK_a = 7.7$.

From the mechanistic point of view, the opposite end of the curve (Fig. 4, acidic pH range) deserves more attention. It is clearly seen that the experimental points deviate slightly but significantly from the theoretical curve. This holds even if the theoretical curve is of higher pK_a (not shown). This deviation is much less than that observed with papain (Polgár, 1973). Nevertheless, a similarly small deviation has already been found with alkylation of the essential thiol group of D-glyceraldehyde-3-phosphate dehydrogenase (Polgár, 1975). The deviation from conventional dissociation in Fig. 4 can be interpreted in terms of an interactive system formed between the thiol group and some other amino acid residue, such as the mercaptide–imidazolium ion-pair in the case of papain.

To pursue the comparison with papain further, we examined the alkylation of bean proteinases with iodoacetate also. With this negatively charged reactant papain displayed a bell-shaped pH–rate profile with pK_a values similar to those found with iodoacetamide (Halász & Polgár, 1977). The rate at the maximum of the curve was two orders of magnitude higher with iodoacetate than with iodoacetamide. This was attributed to an interaction between the carboxylate ion of the charged alkylating agent and the imidazolium ion of the ion-pair at the active site of papain (Halász & Polgár, 1977). It has since been shown that the ion-pair of another cysteine proteinase, ficin, displays the same rate constants and pH-dependence in the reaction with chloroacetate as was found with papain (Brocklehurst *et al.*, 1982). Similarly high rate constants were also obtained in the reactions of chloroacetate with papaya peptidase A and chymopapain (L. Polgár, unpublished work). In the bean proteinase reaction (Fig. 4), however, a bell-shaped pH–rate profile could not be demonstrated, but instead a wide pH-independent region was revealed. This marked deviation from conventional thiol dissociation clearly indicates that some neighbouring group governs the reaction with the thiol group in the pH range studied. In this respect bean proteinase resembles papain. On the other hand, the low reactivity of iodoacetate relative to that of iodoacetamide, which is usual in the reactions with simple thiol compounds such as glutathione (Halász & Polgár, 1977), is unique to the bean proteinase and contrasts with papain. This indicates that in bean proteinase either the mercaptide–imidazolium ion-pair has not been formed, or, if it exists, its geometry and/or its environment is probably different from that of papain. Thiolsubtilisin serves as an example of the latter case. In this enzyme the existence of the mercaptide–imidazolium ion-pair has been demonstrated, but only slight rate enhancement was observed in its reaction with iodoacetate (Halász & Polgár, 1977). The rate constants for the reactions of iodoacetate with papain, thiolsubtilisin and the bean proteinase are $1100 M^{-1} \cdot s^{-1}$, $0.84 M^{-1} \cdot s^{-1}$ and $0.11 M^{-1} \cdot s^{-1}$ respectively. It is remarkable that in this respect bean proteinase resembles thiolsubtilisin, an artificial enzyme lacking true proteinase activity, rather than papain or the related cysteine proteinases.

Summarizing, we conclude that the thiol group of bean proteinase is not a simple functional group, but probably constitutes a part of an interactive system where its nucleophilic reactivity is enhanced. This is indeed characteristic of cysteine proteinases, whose best-known representative is papain. It should be emphasized, however, that there are considerable differences between the bean proteinase and papain, particularly in the pK_a values of acylations, and in the magnitudes of the pH-independent rate constants for alkylations with iodoacetate. Consequently, if the bean enzyme is a cysteine proteinase, as suggested by this

study, its active site exhibits unusual properties, so that it may represent a new variant of such proteinases.

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