The Mutability of Stem Bromelain: Evidence for Perturbation by Structural Transitions of the Parameters that Characterize the Reaction of the Essential Thiol Group of Bromelain with 2,2'-Dipyridyl Disulphide

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Stem bromelain (bromelain, EC 3.4.4.24) resembles papain and ficin in some respects (see Murachi, 1970; Murachi & Takahashi, 1970), in particular in that it contains an essential cysteine residue, the thiol group of which is within 0.5 nm (5 Å) of the imidazole group of a histidine residue (Husain & Lowe, 1968). Bromelain differs from the other two enzymes, however, notably in some of its catalytic properties (see Brocklehurst *et al.*, 1967) and in that it is a glycoprotein (Murachi *et al.*, 1967). Previous work in this laboratory (Wharton, 1969) had demonstrated a marked difference in the catalytic characteristics (see below) of crude and purified bromelain preparations. Our finding that 2,2'-dipyridyl disulphide may be used to detect nucleophilic reactivity in the imidazole-thiol pair of papain (Brocklehurst & Little, 1972) led us to investigate the reaction of this reagent with bromelain. The objects were the detection of (i) a similar nucleophilic system in bromelain and (ii), if this exists, any change in reactivity that the system might undergo during the enzyme purification. Most of the recorded properties of bromelain relate to bromelain prepared essentially by the method of Murachi (Murachi *et al.*, 1964; Murachi, 1970). This method includes chromatography on a cation-exchange resin such as Amberlite CG-50. The product is less than fully active, and usually an 'activator' such as L-cysteine, KCN or dithiothreitol is added to the purified enzyme.

We now report that bromelain preparations that have not been chromatographed on Amberlite CG-50 do not exhibit high reactivity towards 2,2'-dipyridyl disulphide at pH approx. 4. This is true even after treatment of the enzyme with L-cysteine, dithiothreitol, KCN or CaCl₂ (see below), and in the present paper we refer to such preparations as UU-bromelain (untransformed and untransformable). By contrast, bromelain that has been chromatographed on Amberlite CG-50 can exhibit high reactivity towards 2,2'-dipyridyl disulphide in acidic media, as found in the corresponding reaction of papain (Brocklehurst & Little, 1970, 1972). With some preparations treatment with one of the four compounds listed above was necessary after the Amberlite CG-50 chromatography before the high reactivity was generated. These preparations before treatment with one of the reagents we refer to in the present paper as UT-

bromelain (untransformed but transformable). In most cases, however, bromelain exhibited the high reactivity after Amberlite CG-50 chromatography without further treatment, and these preparations as well as those resulting from treatment of UT-bromelain with dithiothreitol etc. we refer to in the present paper as T-bromelain (transformed bromelain).

Materials and methods

Similar results have been obtained with samples of enzyme extracted from crude bromelain powder obtained (i) from Seravac Laboratories (now Miles-Seravac) (Maidenhead, Berks., U.K.), (ii) from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and (iii) as a gift from Dr. Golding of the Dole Co. (Honolulu, Hawaii, U.S.A.). Chromatography columns and eluents were as follows: solutions contained 1 mM-EDTA; unless otherwise stated procedures were as described by Wharton *et al.* (1968); with Amberlite CG-50 (2.8 cm × 85 cm) 0.2 M-K₂HPO₄ containing 1 M-KCl was used; with Sephadex G-100 (2.8 cm × 85 cm) 0.05 M-sodium acetate buffer, pH 5.2, was used; with Sephadex G-25 (2.4 cm × 20 cm) 0.1 M-KCl was used; operations were carried out at room temperature (usually 19–22°C). Other materials and methods used were essentially as described previously (Wharton *et al.*, 1968; Brocklehurst & Little, 1970). The thiol content of all activated activator-free bromelain preparations was found to be 0.4–0.8 mol of SH/mol as measured by titration with (i) 2,2'-dipyridyl disulphide at all pH values in the range 3–10 and (ii) 5,5'-dithiobis-(2-nitrobenzoic acid) at pH 8.0. The thiol content of any one preparation was the same (±10%) at all pH values. The thiol content of crude bromelain before activation was approx. 0.4 mol of SH/mol.

Results and discussion

As with papain (see Brocklehurst & Little, 1970), all three types of bromelain react with 2,2'-dipyridyl disulphide to produce 2-thiopyridone in amount stoichiometric with the thiol content of the protein. Fig. 1 shows plots of apparent (see Brocklehurst & Little, 1972) second-order rate constants, k_{+2} , against pH for reactions of 2,2'-dipyridyl disulphide with UU-bromelain, UT-bromelain and T-bromelain.

Fig. 1. *pH-rate profiles for the reactions of 2,2'-dipyridyl disulphide with (a) UU-bromelain, (b) UT-bromelain and (c) T-bromelain at 25.0°C and 10.1*

Buffers were as follows: pH 2.4–2.8, glycine–HCl; pH 2.8–3.6, sodium formate; pH 3.6–5.6, sodium acetate; pH 5.6–7.2, potassium phosphate; pH 7.2–8.4, tris–HCl; pH 8.4–8.9, sodium borate; pH 9.0–10.6, sodium bicarbonate. The lines are theoretical:

For (a) and (b):

$$k_{+2} = \bar{k}_{+2}/(1 + [\text{H}^+]/K_{a1} + K_{a2}/[\text{H}^+]) + \bar{k}'_{+2}/(1 + [\text{H}^+]/K_{a2} + K_{a3}/[\text{H}^+]) + \bar{k}''_{+2}/(1 + [\text{H}^+]/K_{a3});$$

$$\bar{k}_{+2} = (a) 3.9 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}, (b) 3.03 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}; \bar{k}'_{+2} = (a) \leq 100 \text{ M}^{-1} \cdot \text{s}^{-1}; (b) \leq 100 \text{ M}^{-1} \cdot \text{s}^{-1};$$

$$\bar{k}''_{+2} = (a) 1.26 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}, (b) 0.91 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1};$$

$$\text{p}K_{a1} = (a) 6.15, (b) 6.0; \text{p}K_{a2} = (a) 6.15, (b) 6.0; \text{p}K_{a3} = (a) 8.21, (b) 8.65$$

For (c):

$$k_{+2} = \bar{k}_{+2}/(1 + [\text{H}^+]/K_{a1} + K_{a2}/[\text{H}^+]) + \bar{k}'_{+2}/(1 + [\text{H}^+]/K_{a2} + K_{a3}/[\text{H}^+]) + \bar{k}''_{+2}/(1 + [\text{H}^+]/K_{a3} + K_{a4}/[\text{H}^+])$$

$$+ \bar{k}'''_{+2}/(1 + [\text{H}^+]/K_{a4});$$

$$\bar{k}_{+2} = 1.43 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}; \bar{k}'_{+2} = 4.44 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}; \bar{k}''_{+2} \leq 100 \text{ M}^{-1} \cdot \text{s}^{-1}; \bar{k}'''_{+2} = 4.44 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1};$$

$$\text{p}K_{a1} = 3.5; \text{p}K_{a2} = 3.5; \text{p}K_{a3} = 6.43; \text{p}K_{a4} = 8.54$$

It has not been possible to delineate the sensitive feature that has on occasions permitted the isolation of the intermediate (UT-bromelain) in the transformation of UU-bromelain into T-bromelain. This was isolated on five occasions during a period of 4 months. In many, more recent, experiments, however, the procedure that had previously resulted in the production of UT-bromelain has instead produced T-bromelain. Nevertheless it is important to report the existence of UT-bromelain because our albeit limited study of the conversion of UT-bromelain into T-bromelain throws some light on the nature of the overall transformation of UU-bromelain into T-bromelain (see below).

The feature that most dramatically distinguishes the profiles for the reactions of 2,2'-dipyridyl disulphide with UU-bromelain and with UT-bromelain from that for the reaction with T-bromelain is the value of k_{+2} at pH 3–4. For the reactions with UU-bromelain and UT-bromelain at pH 3.8 and 10.1 at 25°C k_{+2} is approx. $20\text{M}^{-1}\cdot\text{s}^{-1}$ whereas for the corresponding reaction with T-bromelain k_{+2} is approx. $3 \times 10^4\text{M}^{-1}\cdot\text{s}^{-1}$.

Bromelain preparations that have not been chromatographed on Amberlite CG-50 but have been subjected to Sephadex (G-100 and G-25) and/or $(\text{NH}_4)_2\text{SO}_4$ precipitation and/or treatment with L-cysteine, dithiothreitol, KCN or CaCl_2 remain UU-bromelain. Chromatography on Amberlite CG-50 is required to produce either UT-bromelain or T-bromelain. Transformation of UT-bromelain into T-bromelain was effected by treatment for 10 min with 5 mM-dithiothreitol or -L-cysteine or -KCN or with 2 M- CaCl_2 followed by chromatography on Sephadex G-25. This transformation was not effected by 5 mM-L-methionine or guanidinium chloride. Bromelain preparations treated with dithiothreitol, L-cysteine, KCN or CaCl_2 and then chromatographed on Sephadex G-25 before chromatography on Amberlite CG-50 remained UU-bromelain.

It might be expected that L-cysteine, dithiothreitol, CN^- , Ca^{2+} and ion-exchange resins would be capable of disrupting protein structure (see von Hippel & Schleich, 1969). It is suggested that Amberlite CG-50 chromatography either disrupts the structure of UU-bromelain or removes a bound fragment (e.g. a peptide or oligosaccharide) to produce UT-bromelain and that this process allows a subsequent structural change in the protein that provides T-bromelain. The latter change appears to result from interaction with one of the low-molecular-weight compounds listed above or with the Amberlite CG-50 resin.

If this transformation changes the bromelain structure such that the apparent pK_a of the active-centre histidine residue, as determined kinetically by reaction of the enzyme's thiol group with 2,2'-dipyridyl disulphide, changes from 6.0 in UU-bromelain and UT-bromelain to 3.5 in T-bromelain,

the pH-rate profiles in Fig. 1 may be rationalized as follows.

Reaction of T-bromelain. The four ionic states of significant reactivity are designated X, XH, XH_2 and XH_3 to indicate their relative stoichiometries with respect to protons. The overall shape of the profile is similar to that for the analogous reaction of papain (see Brocklehurst & Little, 1972) except that an extra ionization (pK_a 6.0) is apparent in the T-bromelain case. This gives rise to an extra reactive ionic state. The states X and XH may be interpreted, by analogy with the papain reactions, as reactions of the T-bromelain thiolate ion and the T-bromelain thiol-imidazole pair respectively with unprotonated disulphide. It is tentatively suggested that protonation of some unknown group (not the imidazole group of the active-centre histidine residue) in T-bromelain (with apparent pK_a 6.0) to give XH_2 results in a structural change such that the reactivity of the thiol-imidazole pair with unprotonated 2,2'-dipyridyl disulphide is increased. The ionic state XH_3 (analogous to XH_2 in the papain reaction) may then be interpreted in terms of enhancement of the reactivity of XH_2 by protonation on N of the disulphide. The lack of reactivity in XH_4 is interpreted, as with XH_3 in the papain reaction, as resulting from protonation of the histidine imidazole group, which results in loss of nucleophilic character in the cysteine thiol group.

Reactions of UU-bromelain and UT-bromelain. Three reactive ionic states are apparent in these profiles, X, XH and XH_2 . The interpretation of X and XH are as for the reaction of T-bromelain. If the apparent pK_a of the histidine imidazolium ion is 6.0 in the UU- and UT-enzymes, lowering of the pH to below approx. 7.5 will result in protonation of two groups that would be predicted to affect the rates of reaction of the UU- and UT-bromelain thiol groups. These are the active-centre histidine imidazole group (pK_a 6.0) and the other (unknown) group (pK_a 6.0), protonation of which was postulated to increase the reactivity of the T-bromelain imidazole-thiol pair ($\text{XH} \rightarrow \text{XH}_2$). These two protonations could account for the optimum at pH 6.0. One consequence of the higher apparent pK_a of the histidine imidazolium ion in the UU- and the UT-bromelain is that this group will be essentially fully protonated at pH values at which the disulphide is becoming protonated effectively. Thus the high reactivity at pH 3–4 associated with reaction of the thiol-imidazole pair with the protonated disulphide found with T-bromelain and with papain is not observed with the UU- and the UT-bromelain. The values of k_{+2} probably characterize both the binding of the disulphide to the enzyme and the subsequent thiol-disulphide interchange (see Brocklehurst & Little, 1972). Thus the different apparent pK_a values assigned to the histidine imidazolium ions of UU- and UT-bromelain on the one hand and to that of T-bromelain on the other could

result from differences in the intrinsic pK_a values of the imidazolium ions and/or differences in the dissociation constants of the bromelain-2,2'-dipyridyl disulphide complexes.

XH states of the UU-, UT- and T-bromelain reactions. It is important to point out that the reactivities of all the XH states indicated in Fig. 1 are very much lower relative to the reactivities of the corresponding X states than for the analogous reactivity in papain, and are therefore subject to considerable error. Indeed, the results do not preclude the possibility that these reactivities are in fact zero and the uncharacterized protonation $XH \rightarrow XH_2$ is required to generate reactivity in the thiol-imidazole pair.

The differences between UU- and UT-bromelain on the one hand and T-bromelain on the other are reflected in the catalytic properties of these enzyme preparations. The pH- v profiles for the hydrolysis of α -N-benzoyl-L-arginine ethyl ester at $[S_0] < K_m$ catalysed by UU-bromelain and by UT-bromelain approximate to simple 'bells' with pK_a values 6 and 9, whereas the corresponding profile for the hydrolysis catalysed by T-bromelain is a 'complex bell' characterized by an alkaline limb with pK_a 9 and an acid limb with pK_a values 6 and 3.5. The latter is similar in shape to the pH- $k_{cat.}/K_m$ profile for this

hydrolysis reported by Inagami & Murachi (1963). These profiles are similar to those that Wharton (1969) found for catalyses by 'crude' bromelain and 'purified' bromelain respectively.

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