Circular dichroism of stem bromelain : a third spectral class within the family of cysteine proteinases

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Two forms of stem bromelain (EC 3.4.22.4) were isolated from commercial, crude and chromatographically purified preparations of the enzyme by means of gel-filtration and cation-exchange liquid chromatography. These forms possess nearly identical secondary and tertiary structures, as judged from their circular dichroism (c.d.) spectra. The spectral characteristics of stem bromelain suggest that this enzyme belongs to the $\alpha + \beta$ protein class, as other cysteine proteinases do. In agreement with

these results, quantitative estimation of secondary structures yielded amounts similar to those for papain and proteinase Ω . However, the bromelain c.d. curve is clearly distinguishable from those reported for papain and proteinase Ω , on one hand, and that of chymopapain, on the other. Thus it is apparent that there are at least three types of c.d. spectra associated with the family of cysteine proteinases.

INTRODUCTION

Cysteine proteinases comprise a family of enzymes widely distributed in nature. Several members of this family have been isolated from a number of plant sources and in some of these cases the complete amino acid sequence is known. The high identity observed when comparing the sequences of papain [1], actinidin [2], proteinase Ω [3,4], chymopapain [5,6] and stem bromelain [7] is a clear indication that the polypeptide chains of these proteins share a common folding pattern. This has been confirmed, for the first three proteinases mentioned above, by detailed X-ray diffraction studies [8–10]. In addition, papain and proteinase Ω display very similar far-u.v. c.d. spectra, suggesting that the conformations of these proteins in solution are nearly identical [11]. However, the chymopapain c.d. curve notably differs from those of the other papaya proteinases [11], thus posing the interesting question of which molecular characteristics of this enzyme are responsible for the observed spectral discrepancies.

In order to find out whether or not other cysteine proteinases show the c.d. spectrum typical of papain, we studied in this work two of the major forms of stem bromelain. Although some authors have reported that up to six enzyme forms can be obtained from the fractionation of stem bromelain [12], we focused our study on the two most abundant forms which were well separated by means of high-resolution ionic-exchange chromatography [13].

These bromelains had the same M_r values (25400) but they showed distinct electrophoretic mobilities. Their c.d. spectra in the 185–250 nm region, which are nearly identical to each other, look rather similar to those of papain and proteinase Ω ; however, the peculiar negative shoulder at 200 nm, typical of papain and proteinase Ω , is absent in the c.d. curves of the bromelains.

EXPERIMENTAL

Materials

Stem bromelain (crude and chromatographically purified ex-

tracts) and iodoacetamide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and 2-mercaptoethanol was from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Purification of the proteinases from crude stem bromelain

A 0.45 g sample of crude extract was dissolved in 10 ml of phosphate buffer (0.047 M KH_2PO_4 /0.003 M K_2HPO_4 , 3 H_2O , pH 6.0; 5 mM HgCl_2). The suspension was centrifuged for 25 min at 5 °C and 4000 g (r_{av} , 9.75 cm). The supernatant solution was filtered on a 0.42 μm -pore-diam. Millipore filter and applied to a 2.6 cm $\times$ 52 cm column of TSK HW-50 (F) (Merck, Darmstadt, Germany) equilibrated with 0.05 M phosphate buffer. The sample was eluted at a flow rate of 0.8 ml/min and 9 ml fractions were collected. The fractions with higher proteolytic activity were combined and rechromatographed under the same conditions.

Autolysis inhibition

To avoid autolysis, some samples were irreversibly inactivated by reduction and carboxymethylation. Reduction was carried out in 0.1 M 2-mercaptoethanol for 30 min, followed by addition of solid iodoacetamide to give a final concentration of 0.12 M. After stirring for 1 h, the solutions were dialysed against distilled water.

Cation-exchange chromatography

In this study, we used chromatographically purified and crude stem bromelain for the isolation of multiple forms. Crude stem bromelain was first purified by gel-filtration and then by cation-exchange chromatography.

Samples containing 0.7–3.5 mg of stem bromelain dissolved in 0.05 M phosphate buffer (pH 6.0) were injected into a TSK-SP5PW cation-exchange column (0.75 cm $\times$ 7.5 cm) which was installed in a Varian 5020B chromatograph. Some samples were previously reduced and carboxymethylated as described above. The protein fractions were eluted with a linear gradient of

Figure 1 Gel-filtration chromatography of crude stem bromelain on a TSK HW-50 (F) column at pH 6.0

Approx. 0.45 g of crude extract was applied to the column and eluted with 0.05 M phosphate buffer (pH 6.0). The fractions marked by the bars were collected and rechromatographed under the same conditions.

0.15–0.20 M NaCl (slope of 0.0025 M/ml), using the phosphate buffer to create the gradient, at a flow rate of 0.5 ml/min.

Proteolytic activity

The activity was tested at pH 6.0 and 35 °C using casein as substrate; the method was the same as that previously described [14]. One unit of activity is defined as producing a change of 1.0 absorbance unit at 280 nm/min.

Gel electrophoresis

Electrophoresis was performed using a Phast System (Pharmacia LKB). SDS/PAGE was performed as described by Tablero et al. [15]. Band positions were determined by means of a LKB 2202 UltraScan laser densitometer.

For native/PAGE, homogeneous [12.5% (w/v) polyacrylamide] gel slabs (0.45 mm × 43 mm × 50 mm) were used. A constant power of 2.0 W was applied to gels for 40 min at 15 °C.

For isoelectric focusing, Phast gel slabs (0.35 mm × 43 mm × 50 mm; pH range 3–9) were used. Gels were subjected to a constant power of 3.5 W for 40 min, at 15 °C. Fixing, staining and destaining were done according to the procedure described by Neuhoff et al. [16].

C.d. analysis

C.d. spectra were recorded at 25 °C on a JASCO J-500A spectropolarimeter calibrated with (+)-10-camphorsulphonic acid [17]. Measurements in the far-u.v. region (185–250 nm) were made on protein solutions of approx. 0.2 mg/ml in a 0.05 cm pathlength cell. In the aromatic region (250–320 nm), a 1.0 cm pathlength cell and protein concentrations in the range 0.3–0.8 mg/ml were used. Before recording of the spectra, samples were dialysed for 72 h at 4 °C against 0.01 M phosphate buffer (0.006 M KH_2PO_4 /0.004 M K_2HPO_4 , 3 H_2O , pH 7.0).

Figure 2 Cation-exchange chromatography of stem bromelain on a TSK-SP5PW column at pH 6.0

Approx. 3.5 mg of the active material obtained by gel-filtration chromatography (marked by the bars in Figure 1) was injected and eluted with a linear gradient of 0.15–0.20 M NaCl.

Protein concentration was determined spectrophotometrically, employing the absorption coefficient ($A_{1\text{cm}, 280}^{1\%}$) of 20.1 [18]. Mean-residue ellipticity, $[\theta]_{\text{m.r.w.}}$, was calculated using a value of 108 for the molecular mass of a mean residue [7].

RESULTS AND DISCUSSION

Purification of the proteinases

The elution profile obtained upon gel filtration of crude stem bromelain is shown in Figure 1. The fractions marked by the bars were pooled and rechromatographed under the same conditions, where a single peak was obtained (results not shown). We injected 3.5 mg of this material into the TSK-SP5PW column and, as can be seen in Figure 2, three proteolytically active fractions were apparently present in the eluate, but they were poorly resolved. On the other hand, when samples of inactive enzyme were injected into the same column the resolution improved noticeably (Figure 3).

The two fractions indicated in Figure 3 were collected and rechromatographed under the same conditions; in each case a reasonably symmetrical peak was observed at nearly the same elution volume as in Figure 3. These components were designated as stem bromelain 1 (SB1) and stem bromelain 2 (SB2) in order of elution and used for further study. In agreement with these results, the elution profile of chromatographically purified inactive stem bromelain was basically the same as that of Figure 3.

Molecular mass

The relative molecular mass calculated from the position of molecular-mass markers in the same gel (results not shown) was 25400 for both SB1 and SB2. Within the limits of experimental error, this value agrees well with the one derived from the amino acid sequence [7].

Native PAGE and isoelectric focusing

Components SB1 and SB2 were homogeneous by PAGE (results not shown), where they showed different mobilities. SB2 displayed a more basic character, as it migrated slightly faster towards the cathode than SB1. This observation agrees with the chromatographical behaviour shown in Figure 3. Isoelectric focusing results (not shown) indicate that the isoelectric points of

Figure 3 Cation-exchange chromatography of inhibited stem bromelain at pH 6.0

The material obtained by gel-filtration chromatography (marked by the bars in Figure 1) was treated with iodoacetamide (see the text for details) and injected into a TSK-SP5PW column. Protein fractions were eluted with a linear gradient of 0.15–0.20 M NaCl.

stem bromelains are greater than 9, as the proteins focused at positions very near the cathode.

C.d. analysis

C.d. spectra corresponding to the two stem bromelain forms are shown in Figure 4. The curves are almost identical in the complete spectral range studied, indicating that structural differences between SB1 and SB2 are minimal.

In the aromatic absorption region (250–320 nm), c.d. curves of the bromelains are very similar to the spectrum reported earlier by Sakai et al. [19]. On the other hand, in the far-u.v. region these authors have observed bands considerably weaker than those shown in Figure 4. Moreover, Sakai et al. [19] have found a negative band at 214 nm that is displaced to 209 nm in our c.d. spectra. No comparison can be made of the positive band as previous c.d. data were limited to wavelengths above 210 nm.

In general, the spectral characteristics displayed by SB1 and SB2 are typical of $\alpha + \beta$ proteins, the c.d. signal of which is more intense at 208 nm than at 222 nm [20], as is observed in the cases of papain and proteinase Ω [11]. Thus it is probable that bromelain forms may have the same folding pattern shown by other members of the papain family, namely a bilobal structure with all α - and antiparallel β -sheet domains [8]. In concordance

Figure 4 C.d. spectra of stem bromelains

Spectra were recorded in 0.01 M phosphate buffer (pH 7.0) at 25 °C. —, SB1; ---, SB2.

with this proposal, estimation of secondary structure contents in SB1 and SB2 gave results in good general agreement with those for other cysteine proteinases (Table 1), albeit differences in helical content might be of some significance as this type of regular structure is more accurately predicted [21].

In spite of the foregoing similarities, c.d. curves of the bromelains show no evidence of the negative shoulder at 200 nm that clearly appears in papain and proteinase Ω spectra [11]. It might be thought that the cause of this discrepancy lies in the two *N*-acetylglucosamine residues present in the carbohydrate moiety linked to the polypeptide chain of bromelain [22–24]. Indeed, it is well known that mono- or oligo-saccharides containing the 2-acetamido chromophore show intense c.d. bands at wavelengths shorter than 220 nm [25]. However, considering the molar ellipticity of these bands (-4000 to -24000 deg $\cdot$ cm 2 $\cdot$ dmol $^{-1}$) and the approximate 100:1 amino acid to *N*-acetylglucosamine residue ratio, it is likely that the carbohydrate contribution to the c.d. of bromelains does not exceed 3% of the signal at 209 nm (Figure 4). This quantity seems too small to seriously affect the protein spectrum.

According to our results, it is apparent by now that the far-u.v. c.d. spectrum of bromelain represents a third spectral class within the family of cysteine proteinases. The other two types, i.e. those of papain and chymopapain, have been reported before [14,26]. It is not known which structural factors are responsible for this spectral variability, although some possibilities can be advanced: slight differences in pieces of secondary structure or in their arrangements to form supersecondary structures; unlike domain–domain interactions; dissimilar microenvironments of certain aromatic groups or disulphide bonds.

Table 1 Secondary-structure content in papain superfamily proteinases

Fractions of the different structural types in stem bromelain forms were computed from c.d. spectra (185–250 nm) shown in Figure 4, according to the method of Compton and Johnson [21]. Also included are the values for papaya-latex proteinases [11]. The values shown are means $\pm$ S.D.

Secondary structure	SB1	SB2	Papain	Proteinase Ω	Chymopapain
α -Helix	0.23 $\pm$ 0.007	0.23 $\pm$ 0.007	0.27 $\pm$ 0.006	0.27 $\pm$ 0.006	0.31 $\pm$ 0.006
Antiparallel β -sheet	0.18 $\pm$ 0.010	0.19 $\pm$ 0.011	0.12 $\pm$ 0.015	0.14 $\pm$ 0.015	0.07 $\pm$ 0.014
Parallel β -sheet	0.05 $\pm$ 0.008	0.05 $\pm$ 0.008	0.05 $\pm$ 0.012	0.02 $\pm$ 0.012	0.09 $\pm$ 0.013
Turns	0.18 $\pm$ 0.004	0.18 $\pm$ 0.004	0.15 $\pm$ 0.012	0.17 $\pm$ 0.012	0.13 $\pm$ 0.012
Others	0.35 $\pm$ 0.027	0.34 $\pm$ 0.030	0.53 $\pm$ 0.028	0.50 $\pm$ 0.029	0.48 $\pm$ 0.027
Total	1.00	1.00	1.12	1.1	1.08

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