Structural similarity of chymopapain forms as indicated by circular dichroism

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Four chymopapain forms were isolated by high-resolution liquid chromatography on a cation-exchange column. The three major forms possess nearly identical secondary and tertiary structures, as judged from their c.d. spectra; these components showed similar proteolytic activity and M_r values close to that of papain. The fourth isolated component seems to be a mixture of modified proteins.

INTRODUCTION

It is generally accepted that the latex of the papaya (*Carica papaya*) fruit contains at least three different cysteine proteinases [1,2]: papain (EC 3.4.22.2), chymopapain (EC 3.4.22.6) and papaya proteinase A. Papain has been extensively studied and its molecular structure is known in great detail [3]. On the other hand, very little structural information for the other two enzymes is available in the literature. The fraction known as chymopapain has been separated into several forms by cation-exchange chromatography [4–6]; two main components, named chymopapains A and B, seem to be different in the reactivity of the thiol group at the active site [7]. However, Buttler & Barrett [4] have shown that the multiple forms of chymopapain are immunologically identical, suggesting that the chromatographic multiplicity of this proteinase is the result of post-translational modifications [8].

In the present work we have studied the conformational characteristics of four forms of chymopapain by means of c.d. spectroscopy. The results obtained showed that three forms, the most abundant ones, seem to be nearly identical in their polypeptide backbone conformations and in the environment of their aromatic residues.

The estimated content of secondary structures for these chymopapain forms was similar to that of papain. Moreover, we have determined an M_r of approx. 24000 for these enzyme forms; this value is considerably lower than many of the previously reported values [6,9,10], but agrees well with the recent determination made by Kórodi *et al.* [11], and is very close to the M_r of papain (23350) [12].

EXPERIMENTAL

Materials

Commercial chymopapain (substantially free from papain, lysozyme and proteinase A) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The multiple forms of this enzyme were separated on a TSK-CM3SW cation-exchange column (0.75 cm × 7.5 cm) installed in a Varian 5020B chromatograph. A 10 mg portion of com-

mercial chymopapain dissolved in 1 ml of sample buffer (0.031 M- NaH_2PO_4 /0.019 M- Na_2HPO_4 , pH 7.0) was applied to the column. The protein fractions were eluted with a linear gradient of 0–0.70 M- NaCl (0.03 M- NaCl /ml), prepared in the sample buffer, at a flow rate of 0.5 ml/min. We chose these elution conditions on the basis of a study of the effect of pH, flow rate and gradient steepness on the chromatographic resolution of the chymopapain forms (results not shown). Casein was from Merck, Darmstadt, Germany. Guanidinium chloride and the proteins used as standards in the M_r determinations were supplied by Sigma Chemical Co. All other chemicals were of analytical grade.

Proteolytic activity

Assays for proteolytic activity were carried out by the casein assay similar to that previously described [9]. The cysteine proteinases were activated by adding 0.25 ml of enzyme sample (0.05–0.20 mg/ml) to tubes containing 0.75 ml of 0.1 M-sodium phosphate buffer, pH 7.0, that was 0.1 M in cysteine and 0.01 M in EDTA. The tubes were incubated at 35 °C for 5 min, then 1 ml of 1% (w/v) casein solution in the phosphate buffer was added. The tubes were again incubated at 35 °C for exactly 10 min. Addition of 3 ml of 5% (w/v) trichloroacetic acid terminated the reaction. After the tubes had stood for at least 30 min, the reaction mixture was centrifuged and absorbance at 280 nm was measured on the supernatant. One unit of activity corresponded to a change of 1.0 A_{280} unit/min under the conditions described. The specific activity is the activity in units/mg of enzyme [13].

Polyacrylamide-gel electrophoresis

This was performed on horizontal 5% polyacrylamide-gel slabs (2 mm × 250 mm × 115 mm) with 0.05 M-imidazole/ H_3PO_4 buffer, pH 7.0. The electrophoresis was performed at a field strength of 12 V/cm for 2.5 h. Gels were stained and destained as described in ref. [14].

M_r determinations

The gel-filtration method described by Fish *et al.* [15] was used. In this method a protein is denatured in 6 M-guanidinium chloride and its disulphide bonds are

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broken; under these conditions it behaves as a random polypeptide chain whose elution volume is a function of its M_r , regardless of the shape the native structure might have. Proteins, at a concentration of about 0.2 mg/ml, were incubated for 8 h in 6 M-guanidinium chloride/0.1 M-2-mercaptoethanol (pH adjusted to 8.6). Then solid iodoacetamide was added to attain a concentration of 0.12 M; the carboxymethylation reaction was allowed to proceed for 1 h at room temperature. A 10 μ l portion of denatured protein solution was injected into a TSK-2000SW column; the sample was eluted with 6 M-guanidinium chloride at a flow rate of 0.5 ml/min, and the column effluent was monitored spectrophotometrically at 280 nm. The column was calibrated with the following standards: aprotinin (M_r 6500), cytochrome *c* (M_r 12384), lysozyme (M_r 14300), myoglobin (M_r 16950), papain (M_r 23350) and carbonic anhydrase (M_r 29000).

In order to avoid autolysis, the free thiol groups of papain and chymopapain were reduced and carboxymethylated before the denaturation treatment; reduction was carried out in 0.1 M-2-mercaptoethanol, pH 8.6, for 1 h, followed by the addition of solid iodoacetamide in slight excess with respect to 2-mercaptoethanol. After 1 h, the protein was separated from the low- M_r compounds by gel filtration on Sephadex G-25.

C.d.

C.d. spectra were recorded at 25 °C on a JASCO J-500A spectropolarimeter calibrated with (+)-10-camphorsulphonic acid [16]. Measurements in the far-u.v. region (190–250 nm) were made on protein solutions of 0.1–0.2 mg/ml in a 0.10 cm cell. In the near-u.v. region (330–250 nm) a 1.0 cm cell was used, and the protein concentration was in the range 0.15–0.60 mg/ml. Samples used in the c.d. studies were first dialysed for 72 h at 4 °C against 0.03 M-sodium phosphate buffer, pH 7.0, and then diluted to the desired concentration with the same buffer. In all cases protein concentration was determined by using $A_{1\text{ cm}, 280}^{1\%} = 18.3$ [9]. C.d. values are reported as mean residue ellipticity, $[\theta]_{\text{m.r.w.}}$, which was calculated by using M_r 110 per residue.

To estimate the content of secondary structures, the c.d. data were analysed by unconstrained least-squares fitting to the basis spectra of Hennessey & Johnson [17]. Since no constraints were imposed on the analysis, a sum of structures that does not deviate largely from 100% and the absence of large negative values for any of the structural types will indicate that reliable results have been obtained [16,17].

RESULTS AND DISCUSSION

The elution profile of the chromatography of commercial chymopapain on the TSK-CM3SW column is shown in Fig. 1(a). The four fractions marked by the bars (denoted as peaks I–IV) were pooled separately and rechromatographed twice under the same conditions. After the second rechromatography reasonably symmetrical peaks were obtained (Fig. 1b), and the material collected as indicated by the bars was used for further study.

The four isolated components were found to be homogeneous by polyacrylamide-gel electrophoresis (results not shown). In the proteolytic assay against casein, components II, III and IV had specific activities

Fig. 1. Chromatography of commercial chymopapain on a TSK-CM3SW column at pH 7.0

(a) Approx. 10 mg of protein was applied to the column and eluted with a 0.05–0.70 M-NaCl gradient (----) as described in the Experimental section. (b) The fractions collected in (a) (marked by the bars) were concentrated to different extents and rechromatographed under the same conditions.

of 1.27, 1.30 and 1.40 units/mg respectively; these values compare well with the specific activity of 1.07 units/mg reported for chymopapain A [10] and 3.4 units/mg for chymopapain B [6]. On the other hand, component I had only 0.15 unit/mg.

M_r determinations

Gel-filtration experiments in 6 M-guanidinium chloride (Fig. 2) showed that components II and IV are eluted as single symmetrical peaks of nearly identical elution volumes (7.42 and 7.43 ml); for these two components an M_r of 24400 was calculated from the calibration curve of the TSK-2000SW column. Component III was also eluted as a single peak, corresponding to an M_r of 24100. In contrast, in the elution profile of component I two peaks were observed corresponding to M_r values of 24000 and 13100, in a ratio of 1:1.2. The M_r values determined in the present work for components II, III and IV are in very good agreement with the value of 24200 obtained by Kórodi *et al.* using SDS/polyacrylamide-gel electrophoresis [11]. Furthermore, our results confirm that the chymopapains are formed by a single polypeptide chain, since a single peak is observed in gel filtration after denaturation and breaking of the disulphide bonds. On the other hand, the presence of two broad peaks in the elution profile of component I suggests that this minor component is a mixture of hydrolytically transformed proteins.

C.d. studies

Fig. 3 shows the c.d. spectra of the isolated chymopapain forms. The c.d. curves for components II–IV

Fig. 2. Determination of M_r values of the four chymopapain components

The fractions isolated in Fig. 1(b) were chromatographed on a TSK-2000SW column eluted with 6 M-guanidinium chloride. The samples were previously treated as described in the Experimental section.

are very similar in both far-u.v. and near-u.v. regions, whereas the spectrum of component I is notably different. Since the far-u.v. region of a protein c.d. spectrum is mainly due to the secondary conformation [17], whereas the near-u.v. region reflects the asymmetrical environment of aromatic residues [18], it is clear that the three major components of chymopapain possess nearly identical secondary structure, as well as tertiary structure around the aromatic side chains. Moreover, this similarity in certain regions of the tertiary structure seems to extend to papain, whose c.d. spectrum [19] shares the main features of the curves in Fig. 3, namely a negative band around 300 nm and a progression of positive bands at 288, 279 and 273 nm.

Fig. 3. C.d. spectra of the four chymopapain components

Spectra were recorded at pH 7.0 and 25 °C. In the far-u.v. region (190–250 nm) dots represent the curves reconstructed from the results of the analysis for secondary-structure estimation.

In order to estimate the content of secondary structures in the chymopapain components, we analysed their c.d. spectra (190–250 nm) in terms of the basis spectra reported by Hennessey & Johnson [17]. The results obtained (Table 1) indicated that the analysis was satisfactory for components II, III and IV, since the sum of structure fractions was close to unity; besides, the curves reconstructed from the results of this analysis showed a very good fitting to the experimental spectra (Fig. 3). In the case of component I, a sum of fractions of 0.574 indicates that the analysis was poor. Nevertheless, the c.d. spectrum of this component looks very similar to the other spectra but with a lower intensity. This fact, as well

Table 1. Secondary-structure content in the chymopapain components

Structural contents, which are expressed as fractions, were estimated from the c.d. spectra (190–250 nm) of the components indicated in Fig. 1 as described in the text. Papain data (from ref. [17]) are included for comparative purposes. The values for components I–IV are given as means $\pm$ s.d.

Sample	α -Helix	Antiparallel β -sheet	Parallel β -sheet	Turns	Others	Total
Component I	0.140 ± 0.006	0.098 ± 0.008	0.020 ± 0.006	0.109 ± 0.003	0.207 ± 0.021	0.574
Component II	0.224 ± 0.006	0.119 ± 0.009	0.055 ± 0.007	0.164 ± 0.003	0.395 ± 0.024	0.956
Component III	0.197 ± 0.007	0.096 ± 0.010	0.062 ± 0.008	0.140 ± 0.004	0.370 ± 0.027	0.865
Component IV	0.228 ± 0.006	0.113 ± 0.009	0.062 ± 0.007	0.167 ± 0.003	0.430 ± 0.023	1.001
Papain	0.240	0.080	0.070	0.150	0.440	0.980

as the low caseinolytic activity and the results observed in the M_r determination for component I, suggest that it could be a mixture of molecules with near-native structure, and autolysis fragments with an unknown, but small, contribution to the total c.d. signal.

As shown in Table 1, the three major chymopapain components are structurally closely related to papain, with only slight differences in the α -helix and β -sheet contents. These results, taken together with the similarity in the aromatic c.d. spectra mentioned above, give support to the idea of a common evolutionary origin and a similar three-dimensional structure for the cysteine proteinases, as has been suggested on the basis of amino acid sequence information [20,21].

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