A Spectrophotometric Method for the Detection of Contaminant Chymopapains in Preparations of Papain

SELECTIVE MODIFICATION OF ONE TYPE OF THIOL GROUP IN THE CHYMOPAPAINS BY A TWO-PROTONIC-STATE REAGENT

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A simple spectrophotometric method for the detection of chymopapains A and B, contaminants in some preparations of papain (EC 3.4.22.2), was devised. If the amount of rapidly reacting thiol in the preparation, as determined by increase in \( A_{343} \) consequent on reaction with the two-protonic-state thiol titrant, 2,2'-dipyridyl disulphide, is greater at pH8–9 than at pH4, contamination by the chymopapains is indicated.

The isolation of papain (EC 3.4.22.2) from the commercially available latex of Carica papaya involves the removal of three other thiol proteinases, namely chymopapain A, chymopapain B and papaya peptidase A (see Ebata & Yasunobu, 1962; Schack, 1967; Kunimitsu & Yasunobu, 1970; Robinson, 1975). It is a common practice in the isolation of papain to precipitate a 'papain-rich' fraction from latex solutions by the (NH\(_4\))\(_2\)SO\(_4\) fractionation method of Kimmel & Smith (1954), which has been described also by Arnon (1970). The efficiency of this fractionation step is very sensitive to protein concentration (Baines et al., 1978) and, because improvements in the commercial production of papaya latex (see, e.g., Jones & Mercier, 1974) have resulted in products with much higher solubilities, the method of Kimmel & Smith (1954) now produces crystalline preparations of papain that are contaminated with the chymopapains unless the protein concentration is carefully controlled.

Although papain can be separated from contaminant chymopapains by chromatography on SP (sulphopropyl)-Sephadex it is simpler to make sure that the chymopapains have been removed in the salt fractionation steps. The present paper reports a simple and rapid spectrophotometric method for the detection of contaminant chymopapains in papain preparations. This method facilitates the establishment, for a particular batch of latex, of conditions for the successful and reliable separation of the chymopapains from papain by salt fractionation. If the thiol content of a preparation of papain as determined by 'instantaneous' spectrophotometric titration with 2,2'-dipyridyl disulphide is the same when the titration is performed at pH4 as when it is performed at pH8–9, the preparation is probably free from chymopapains. A thiol content determined at pH8–9 that is higher than that determined at pH4 is characteristic of contamination by chymopapains.

Materials and Methods

Many of the materials and general methods have been described previously (Brocklehurst & Little, 1973; Shipton & Brocklehurst, 1978).

Chymopapains A and B and papaya peptidase A were prepared by chromatography on SP-Sephadex-50 by the method of Robinson (1975). Buffers were prepared as described by Dawson et al. (1969) and by Long (1971).

Routine evaluation of contamination of papain samples by the chymopapains

The papain sample is activated by incubation as a solution (70\( \mu \)M-protein) in KH\(_2\)PO\(_4\)/NaOH buffer, pH7.8, 10.1, containing 20\( \text{mm} \)-L-cysteine and 1\( \text{mm} \)-EDTA for 30min at room temperature (approx. 22°C). The protein is isolated by gel filtration on Sephadex G-25 and its thiol content is determined by reaction with 2,2'-dipyridyl disulphide at pH4 (formic acid/NaOH) and at pH8–9 (Tris/HCl; glycine/NaOH).

The \( A_{343} \) of a 1cm sample containing buffer (1.0ml to give final \( I_{0.1} \), water (1.5–\text{ml}) and enzyme (\text{ml} to give final concn. approx. 10\( \mu \)M) is balanced on the 0–0.1 \( A \) range of a spectrophotometer against that of a reference cell containing buffer (1.0ml) and water (1.5ml), and a baseline is recorded (chart speed 50–100s/in). After the addition of 2,2'-dipyridyl disulphide (0.5ml to give final concn. 100–250\( \mu \)M) to the reference cell, the same addition is made to the sample cell to start the
reaction and recording is continued until reaction appears to be complete. It is not necessary to record for more than approx. 1 min at pH 8-9. If the essentially instantaneous increase in \( e_{343} \) at pH 4 is less than that observed at pH 8-9, it is necessary to record for approx. 30 min to monitor the complete progress curve for the reaction of the slowly reacting thiol group of the chymopapains, which has no analogue in papain. The value of \( e_{343} \) for 2-thiopyridone, the chromophoric product released in the thiol titration, may be calculated by using:

\[
e_{343} = \frac{8.08 \times 10^3}{1 + \frac{1.58 \times 10^{-10}}{[H^+]} \text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}}
\]

(see Brocklehurst & Little, 1973; Stuchbury et al., 1975).

Results and Discussion

The possibility of detecting chymopapains in samples of papain by the simple and rapid spectrophotometric method here reported derives from the presence in each of the chymopapains of a thiol group with reactivity characteristics that are markedly different in acidic media from those of the papain thiol group.

Thiol groups in environments like that of the papain active centre are readily distinguished from thiol groups in many other types of environment by using as thiol titrants and reactivity probes reagents that exhibit different electrophilic reactivities in different protonic states (Brocklehurst, 1974).

A convenient two-protonic-state thiol titrant that permits such differential analysis is 2,2'-dipyridyl disulphide (see, e.g., Brocklehurst & Little, 1973; Shipton & Brocklehurst, 1978). The second-order rate constants \( (k) \) that characterize the reactions of this reagent with the thiol groups of papain, the chymopapains and papaya peptidase A at three pH values are given in Table 1. For the purpose of the present paper it is useful to classify the thiol reactivities as 'high' or 'low' according to whether they provide for 'instantaneous' reaction when using excess of 2,2'-dipyridyl disulphide (concentration 100-250 \( \mu \)M) and conventional spectral analysis (absorbance recorded after 20-60 s). When \( [2,2' \text{-dipyridyl disulphide}] = 250 \mu \text{M, } \) half-lives of reaction are complete after 20 s when \( k \approx 680 \text{m}^{-1}\cdot\text{s}^{-1} \).

In these terms the single thiol group present in papain exhibits high reactivity at pH 4, 8 and 9, as does the single thiol group present in papaya peptidase A. Both chymopapains possess two thiol groups per molecule and in each case one thiol group per molecule is of high reactivity at pH 4, 8 and 9. The other type of thiol group present both in chymopapain A and in chymopapain B exhibits only very low reactivity at pH 4 whereas it exhibits high reactivity in alkaline media. Thus, whereas all of the six thiol groups listed in Table 1 react 'instantaneously' with 2,2'-dipyridyl disulphide at pH 8-9, under the conditions stated only four of them react 'instantaneously' at pH 4; one thiol group per molecule of each of the chymopapains reacts only very slowly. The thiol contents of papain preparations that are contaminated with one or both of the chymopapains, therefore, are higher when determined by 'instantaneous' titration with 2,2'-dipyridyl disulphide at pH 8-9 than when determined at pH 4.

It would not be possible to detect papaya peptidase A as a contaminant in papain preparations by this simple type of spectroscopic titration because the reactions of both enzymes with 2,2'-dipyridyl disulphide are 'instantaneous' both at pH 4 and at pH 8-9. Fortunately, the very high solubility of papaya

<table>
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<th>Enzyme</th>
<th>pH</th>
<th>4.0</th>
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<th>9.0</th>
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<td></td>
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<td>1210</td>
<td>1400</td>
</tr>
<tr>
<td>Chymopapain A (fast phase)</td>
<td></td>
<td>2610</td>
<td>12900</td>
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<tr>
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<td>11400</td>
<td>4750</td>
<td>21100</td>
</tr>
<tr>
<td>Chymopapain A (slow phase)</td>
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<td>18</td>
<td>1670</td>
<td>approx. 15600</td>
</tr>
<tr>
<td>Chymopapain B (slow phase)</td>
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<td>505</td>
<td>967</td>
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peptidase A in (NH₄)₂SO₄ solutions suggests that this enzyme is an unlikely impurity in crystalline preparations of papain. This view is supported by the results of analysis by chromatography on SP-Sephadex of papain preparations that did exhibit unequal values of thiol titre with 2,2′-dipyridyl disulphide at pH 4 and 9. The main contaminant in these preparations was chymopapain A. Chymopapain B was present in smaller amount and the presence of papaya peptidase A could not be detected.

Another impurity that could provide unequal values of thiol titre with 2,2′-dipyridyl disulphide at pH 4 and 9 is denatured papain that still retains a thiol group (Brocklehurst & Little, 1973). Denatured papain could be distinguished from the chymopapains as the source of contamination by subjecting the preparation to covalent chromatography by thiol–disulphide interchange (Brocklehurst et al., 1973, 1974) at pH 4. This procedure separates papain from denatured papain, but not from the chymopapains, since these enzymes each possess one thiol group with high reactivity to the pyridyl disulphide gel used in the chromatographic process in addition to the thiol group that exhibits only low reactivity to 2,2′-dipyridyl disulphide. This type of denatured papain, however, does not appear to be a commonly encountered impurity in papain preparations. In our experience, papain preparations that have been freed from the chymopapains have always been characterized by the same thiol titre towards 2,2′-dipyridyl disulphide at pH 4 and 9.

A convenient procedure for the preparation of fully active papain may be summarized as follows.

(i) A crystalline 'papain' preparation is made essentially as described by Kimmel & Smith (1954), with due control of the protein concentration in the (NH₄)₂SO₄ fractionation step (Baines et al., 1978); this preparation should contain papain itself, the papain–cysteine mixed disulphide (Sluyterman, 1967; Klein & Kirsch, 1969) and papain-sulphinic acid (Dreuth et al., 1975).

(ii) The papain–cysteine mixed disulphides reduced to papain by treatment with 20 mm-L-cysteine for 30 min at pH 7.8, and the mixture of proteins is separated from low-molecular-weight material by gel filtration on Sephadex G-25.

(iii) The thiol content of the freshly prepared activated activator-free preparation is determined by spectrophotometric titration with 2,2′-dipyridyl disulphide at pH 4 and at pH 8–9.

(iv) Preparations that are shown to be free from the chymopapains by this test may safely be subjected to covalent chromatography (Brocklehurst et al., 1973, 1974; Stuchbury et al., 1975), which then provides fully active papain containing 1 mol of thiol/mol of protein with high reactivity towards 2,2′-dipyridyl disulphide both at pH 4 and at pH 8–9.

It is important to evaluate the crystalline papain preparation for possible contamination by the chymopapains as soon as possible after its preparation. This is because the chymopapain thiol group with low reactivity towards 2,2′-dipyridyl disulphide in acidic media appears to undergo oxidation much more rapidly than the other thiol group of these enzymes and this destroys the basis of the detection method here reported. When the wet crystalline protein is stored at 4°C the selective oxidation of the thiol group that is of interest in the present connection is 50% complete after approx. 7 days. During this period of storage, oxidation of the thiol group that exhibits high reactivity in acid media is negligible.

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References