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Spectroscopic studies of stability of papain in aqueous organic solvents

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Abstract

The effects of different concentrations of the water-miscible organic solvents ethanol, 1,4-dioxane, tetrahydrofuran (THF) and acetonitrile (ACN) on the stability of papain in aqueous solution were studied. Papain exhibited high stability in aqueous ACN, ethanol and 1,4-dioxane. Decreases in the activity of the enzyme were observed at organic solvent concentrations above 60%. THF caused a dramatic reduction in activity even at low concentrations (5–10%). The solvent-induced structural changes were followed by means of circular dichroism (CD) and intrinsic fluorescence spectroscopy measurements. The decreases in enzyme activity at 90% THF or 90% 1,4-dioxane were accompanied by the loss of the tertiary structure. However, at 90% ethanol and 90% ACN, papain exhibited an increased amount of the α-helical conformation, with little change in the tertiary structure.
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Keywords: Papain; Stability; Aqueous organic solvents; Intrinsic fluorescence; CD spectroscopy

1. Introduction

Enzymatic reactions in aqueous organic solvent systems have recently received a great deal of attention [1,2]. Hydrolytic enzymes, and especially the proteases, have been intensively studied because of their potential applications in the synthesis of biologically active peptides [3,4]. Papain has proven to be a versatile protease for the synthesis of a variety of peptides, due to its broad substrate specificity [5,6]. Enzymes are generally less stable in aqueous organic solvents than in water, and much work is still required to optimize the conditions for enzymatic catalysis in these systems. The attainment of higher levels of catalytic activity of enzymes is one of the major challenges in non-aqueous enzymology. Water-miscible organic solvents can interact with both enzymes and the water molecules associated with the protein structure and can reduce the activity and/or stability of the enzymes. We earlier studied the effects of various organic solvents (ethanol, 1,4-dioxane and acetonitrile) at different concentrations on the kinetic parameters and conformational stability of hydrolytic enzymes, and found significant changes in the secondary structures of these enzymes [7,8].

Papain is a highly stable enzyme, one of the sulfhydryl proteases of *Carica papaya* latex. It has been well studied because of its interesting molecular structure and its many industrial applications. Its molecular structure consists of two distinct domains, with the active site in the groove between the domains. The first domain (residues 1–110) contains mainly α-helix, while the second domain (residues 111–212) has a large content of antiparallel β-sheet and a lesser amount of α-helix. Three of the five tryptophan (Trp) residues are located in the first domain (Trp7 in the β-sheet, Trp26 and Trp69 in the α-helical segments), and two (Trp177 and Trp181) in the coil region of the second domain [9]. The enzyme possesses seven cysteine residues, but only one of them, Cys25, provides the free thiol group of the active site, the remainder forming disulfide bridges [9]: Cys22–Cys63, Cys56–Cys95 and Cys153–Cys200. The structural stability of each lobe is at least partly due to the hydrophobic core, to which many distinct amino acid side-chains contribute. Water molecules present in ice-like networks play an important role in the stability of the enzyme, especially at the domain–domain interface [10].

The aim of the present work was to study the effects of water-miscible organic solvents [ethanol, 1,4-dioxane, tetrahydrofuran (THF) and acetonitrile (ACN)] in aqueous solution on the activity and conformational stability of papain. The alterations in the secondary and tertiary structures of the enzyme were followed

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by means of fluorescence spectroscopic and far- and near-UV circular dichroism (CD) measurements.

2. Materials and methods

2.1. Materials

Papain (EC 3.4.22.2) was a twice-crystallized enzyme, purchased from Sigma–Aldrich. Its specific activity was 14.0 units/mg protein. All other chemicals were reagent grade products of Reanal.

2.2. Assay of enzyme activity

The proteolytic activity of papain, with casein as substrate, was determined according to Arnon [11]. The reaction mixture (2 ml) contained 15 mM disodium citrate/NaOH buffer (pH 6.2), 0.67% casein and 420 μl of enzyme sample. Incubation was performed for 5 min at 25 °C and stopped by the addition of 1.5 ml of 10% trichloroacetic acid. The concentration of the acid-soluble digestion product in the supernatant was determined according to Lowry et al. [12].

2.3. Stability tests

The stability tests in the different aqueous organic solvents at 25 °C involved the use of distilled water at pH 6.2, with an enzyme concentration of 0.1 mg/ml. The samples (2 ml) were incubated for 20 min or 2 h and the residual activities were determined by using the standard method described above.

2.4. Fluorescence spectroscopy

The fluorescence spectrum of papain was monitored on a Hitachi F-2500 FL spectrofluorimeter. The spectrofluorimeter automatically provided corrected spectra by comparison with a 1 nM standard solution of rhodamine B to avoid changes in the lamp output and instrument geometry. Enzyme samples were previously incubated for 2 h and the final protein concentration was 0.1 mg/ml. To estimate the direct effects of organic solvents on Trp fluorescence, *N*-acetyl-L-Trp-ethyl ester was tested as a model compound at 25 mM. Three spectra were accumulated and averaged for each sample. For fluorescence of the Trp, excitation was performed at 292 nm and emission spectra were recorded in the interval 300–450 nm, with slit widths of 2.5 and 5 nm for excitation and emission, respectively [13,14]. The maximal fluorescence intensity of papain was normalized. The maximal fluorescence intensity of papain, measured in water, was taken as one unit in the estimations of the effects of the organic solvents.

2.5. Circular dichroism spectroscopy

CD spectra were recorded in the far-UV range, from 190 to 250 nm, in an optical cell with a pathlength of 0.02 cm, and in the near-UV range, from 250 to 300 nm, in a 1-cm cell on a Jobin-Yvon Mark VI dichrograph at 25 °C. Four spectra were accumulated and averaged for each sample. The concentrations of the protein solutions were adjusted to 0.1 mg/ml (pH 6.2) for the far-UV measurements, and to 0.55 mg/ml for the near-UV measurements. Mean residue ellipticity, $[\Theta]_{MR}$, was expressed in deg cm^2 dmol^{-1}, using a mean residue weight of 110. Percentages of secondary structures were calculated by applying the Provencher and Glöckner curve-analyzing algorithm [15].

3. Results

3.1. Effects of organic solvents on stability of papain

The effects of different concentrations of ACN, 1,4-dioxane and ethanol on the activity of papain in aqueous solution are shown in Fig. 1. Although these organic solvents have different chemical characters (ACN is polar, ethanol is protic, and 1,4-dioxane is aprotic), their effects on the activity of papain are similar. At up to 60% organic solvent content, practically no decrease in activity was observed, but at higher concentrations the enzyme activity did decrease. At 90% ACN, only 36% of the initial activity was preserved, as compared with 75% in 90% ethanol and 82% in 90% 1,4-dioxane. In 5% THF, the enzyme activity decreased dramatically (by 38%) during the 20-min incubation. Fig. 2 presents the rate of inactivation of papain in water, in 90% ethanol and in 90% ACN at 25 °C. At this high concentration, 43 and 55% of the enzyme activity, respectively, was retained after incubation for 2 h. It is known that the internal water molecules play an important role in maintaining the structural stability of papain [10]. Replacement of these water molecules by organic solvent molecules could well be responsible for such changes in activity. It has been observed that the hydrolytic activity and the number of active sites of papain were decreased in aqueous organic solvents (aliphatic alcohols and dimethylformamide) [16]. To establish whether the changes in the catalytic activity of papain in aqueous organic solvents are accompanied by alterations in the secondary and tertiary

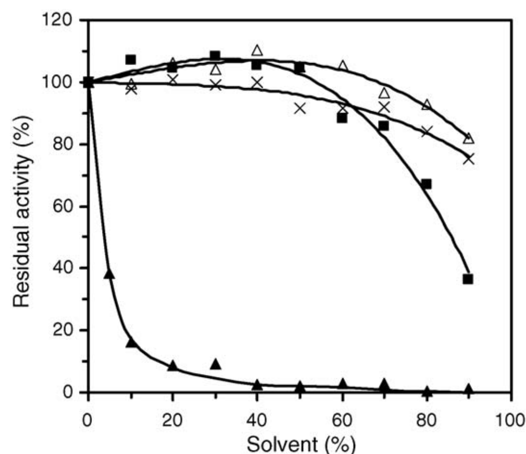

Fig. 1. Effects of different concentrations of acetonitrile (■), 1,4-dioxane (△), tetrahydrofuran (▲) and ethanol (×) on papain activity in aqueous solution. Experiments were carried out at 25 °C with incubation for 20 min, at an enzyme concentration of 0.1 mg/ml.

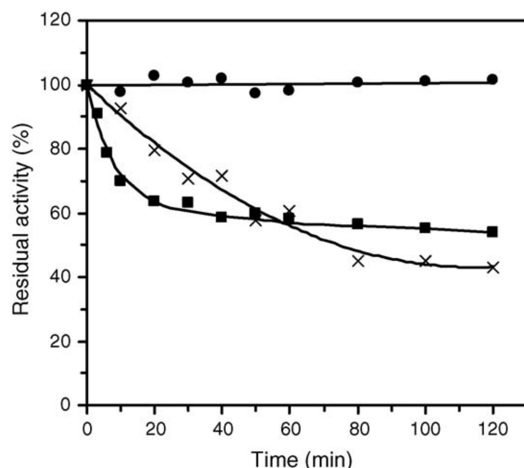

Fig. 2. Time dependence of papain stability in water (●), in 90% ethanol (×) and in 90% acetonitrile (■). The residual hydrolytic activities were determined by the standard methods (see Section 2).

structures of the enzyme, fluorescence and CD spectroscopic measurements were performed.

3.2. Fluorescence spectroscopic studies

The fluorescence properties of the Trp in papain were studied in aqueous ethanol, ACN, THF and 1,4-dioxane. Both the protein conformation in the vicinity of the Trp residues and the solvent polarity contribute, although in various proportions, to the changes in the characteristic fluorescence parameters, the normalized maximal intensity (I_{max}) and the maximal emission wavelength (λ_{max}). It is well known that the fluorescence intensity of Trp is quenched by polar solvents and several other compounds containing proton-donor groups [17]. To estimate the solvent effect, we recorded the fluorescence spectra of *N*-Ac-L-Trp-ethyl ester at different organic solvent concentrations.

Fig. 3 shows the I_{max} values of *N*-Ac-L-Trp-ethyl ester and papain, measured at the appropriate λ_{max} in ethanol and 1,4-dioxane. It can be seen that, with increasing organic solvent concentration, i.e. with decreasing polarity of the environment, the maximal intensity of the fluorescence of N-Ac-L-Trp-ethyl ester increases in accordance with the above statement. The I_{max} values of Trp residues in papain change in a similar way at lower solvent concentrations, while at higher solvent contents considerable deviations can be observed, these being the least pronounced in ethanol and ACN. I_{max} continuously increases with increasing ethanol and ACN concentration, whereas in 1,4-dioxane and THF a maximum can be observed at 70 and 60%, respectively, after which I_{max} decreases (data not shown for ACN and THF).

The λ_{max} value (350 nm in water) of *N*-Ac-L-Trp-ethyl ester showed a gradual blue shift with increasing concentration of any organic solvent, reaching its maximal values at 90% solvent content (Table 1). The dependence of the λ_{max} of papain on the organic solvent concentration was more complex (Fig. 4). In ethanol, ACN and THF, λ_{max} exhibited a continuous blue shift, whereas in 1,4-dioxane the increase in I_{max} was accompanied by a red shift, followed by a blue shift with decreasing I_{max} values (Table 1). Similar findings have been reported for papain fluorescence at pH 2 in halogenated alcohols in aqueous 1,1,1,3,3,3-hexafluoroisopropanol or 2,2,2-trifluoroethanol [18,19].

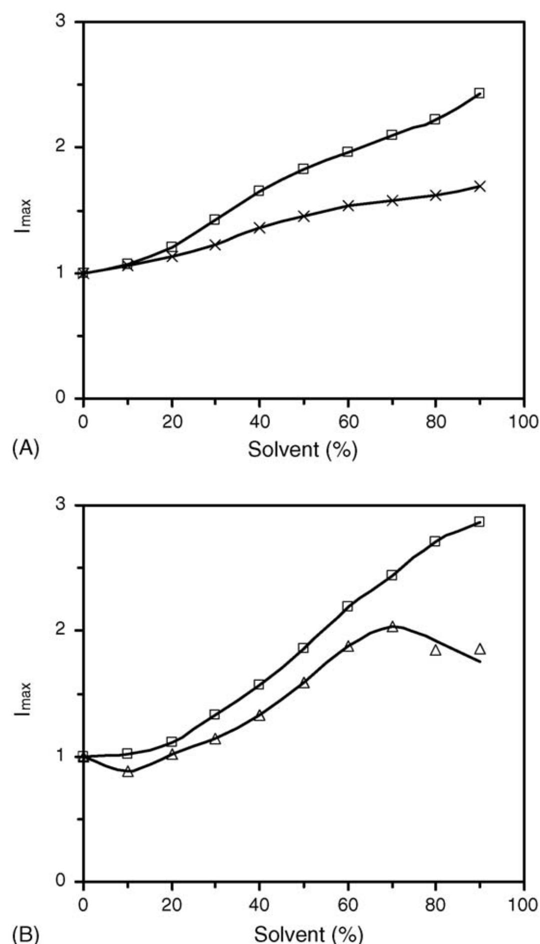

Fig. 3. Effects of different concentrations of ethanol (A) and 1,4-dioxane (B) on I_{max} values of *N*-acetyl-L-Trp-ethyl ester (□) and papain (× and △) measured at the appropriate λ_{max}.

3.3. Far-UV CD studies

Papain is an $\alpha + \beta$ protein with separate α-helix and β-sheet-rich regions. The CD spectra of such proteins in water are dominated by the α-helix portion, exhibiting negative bands at around 208 and 222 nm and a positive band below 195 nm [20]. In the case of papain, an additional negative peripheral band appears at 200 nm [21]. Fig. 5 depicts the far-UV CD spectra of papain in water, at 90% ACN and at 90% ethanol. Because of the high solvent absorbance, CD spectra were not measured in this region for 1,4-dioxane and THF. In water, the enzyme displays a spectrum characteristic of the native state. In 90% ethanol and 90% acetonitrile solutions, the intensities of the negative CD bands increase, with no significant difference between the two solvents. The spectral changes reflect the increase in the α-helical conformation: the Provencher–Glöckner curve-analyzing algo-

Table 1
λ_{max} values of *N*-Ac-L-Trp-ethyl ester and papain fluorescence at 90% solvent content after 2 h incubation

Compound	λ_{max} (nm)				
	Water	Ethanol	Acetonitrile	1,4-Dioxane	Tetra-hydrofuran
N-Ac-L-Trp-ethyl ester	350	338	336	333	333
Papain	339	333	331	333	332

rithm yielded 15, 22 and 20% α-helix content in water, ethanol and ACN, respectively. This conformational change probably indicates the formation of an intermediate (I) state between the native (N) and unfolded (U) states, which is usually characterized by an increased β-sheet [13,14,22,23] or α-helix [18,19] content, depending on the methods and solvents applied.

3.4. Near-UV CD experiments

In the spectral range 250–300 nm, papain in water has a positive CD spectrum with a definite fine structure (Fig. 6) due to the asymmetrically perturbed electronic transitions of the aromatic chromophores and the intrinsically asymmetric disulfide groups [24]. Since the aromatic residues are involved in different interactions, their near-UV contributions vary strongly both in intensity and in sign. Moreover, the positions of the bands depend on the polarity of the environment: an increasingly non-aqueous environment (such as an aqueous organic solvent or the interior of the molecule) produces a red shift [24]. As may be seen in Fig. 6A, the intensities and positions of these bands do not change significantly on increase of the ethanol concentration up to 50%, except at the short-wavelength edge of the spectrum, possibly due to the environmental changes around the disulfide chromophores (below 260 nm). Somewhat greater spectral changes are observed in 70–90% ACN, with a concomitant red shift of the band at 270–273 nm (Fig. 6B). In aqueous 1,4-dioxane (Fig. 6C) and in aqueous THF solution (Fig. 6D), the CD spectra exhibit similar behavior in response to the solvent concentration. The intensity of the CD spectra gradually decreases in the concentration range 30–70%, and at 90% solvent content the low-intensity spectrum completely lacks the bands typical of native papain. Similar spectral changes in the near-UV spectrum of papain have been observed at higher temperature [22] or on acid denaturation [13,23] or in the presence of denaturants [14] as a result of unfolding of the enzyme.

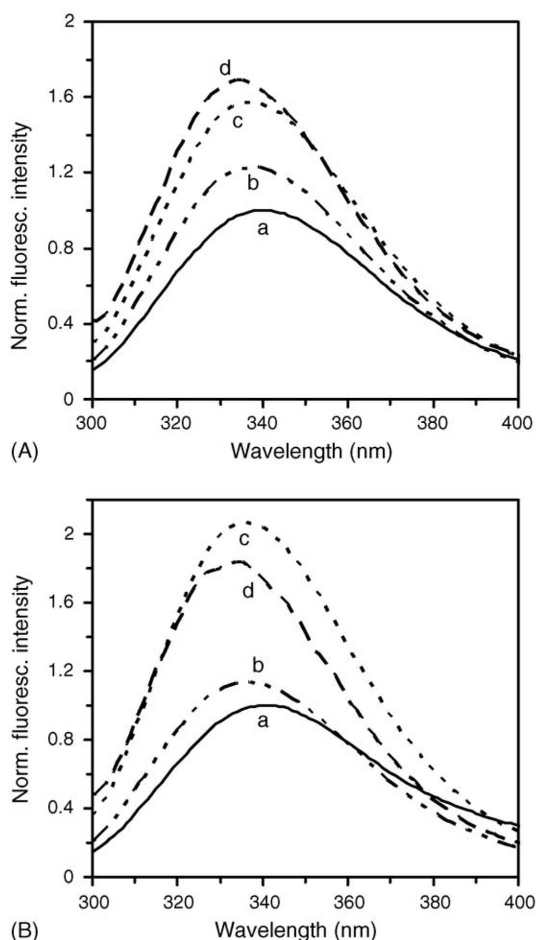

Fig. 4. Spectra of papain fluorescence at different concentrations of ethanol (A) and 1,4-dioxane (B). Water (a), 20% (b), 70% (c) and 90% (d) organic solvent.

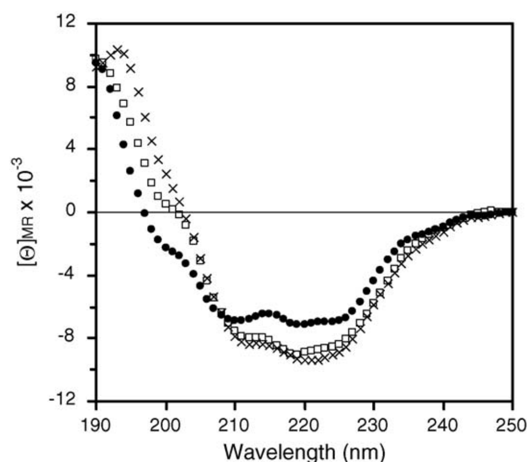

Fig. 5. Far-UV CD spectra of papain in water (●), in 90% ethanol (×) and in 90% acetonitrile (□).

4. Discussion

The effects of the organic solvents on the activity of papain in aqueous solution were different and seemed not to be correlated either with the polarity/hydrophobicity of the solvents or with the overall structure of the enzyme. THF proved to be the most destabilizing solvent for papain.

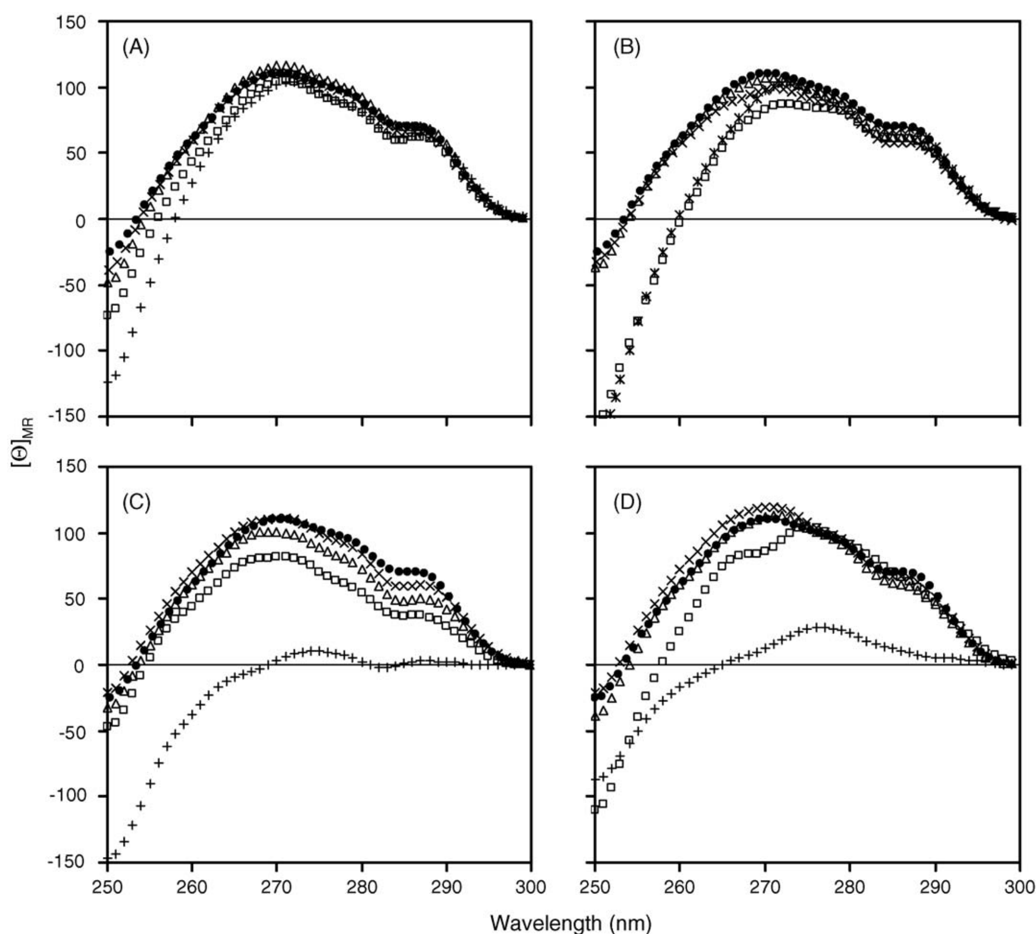

Fig. 6. Near-UV CD spectra of papain in aqueous ethanol (A), acetonitrile (B), dioxane (C) and tetrahydrofuran (D) at different solvent concentrations: 0 (●), 30% (×), 50% (△), 70% (□) and 90% (+) (for acetonitrile, the spectrum at 80% (*) is shown instead of 90%).

In all the organic solvents investigated, the similar responses of the I_{max} of the model compound *N*-Ac-L-Trp-ethyl ester and papain to increasing solvent concentration in the range 0–70% clearly indicate that the changes observed in the I_{max} of papain in this region are not due to change in the protein structure, but merely reflect the effects of the solvent molecules on the Trp residues located on the surface of the protein (Fig. 3). Indeed, the near-UV CD spectra (Fig. 6) reveal that papain does not undergo any significant structural change up to 70% solvent concentration, particularly in ethanol. At higher organic solvent content (from 50–70% to 90%), the I_{max} values decrease (except in ethanol), possibly because of the loss of the tertiary structure, and I_{max} is governed by the dual effect of the solvent polarity and the protein conformation. On the basis of the changes in the tertiary structure of papain, the effects of the solvents can be divided into two groups.

4.1. Structural and catalytic behavior of papain in ethanol and acetonitrile

With increasing concentration of ethanol and ACN, the I_{max} of Trp in papain continuously increases, to the accompaniment of a blue shift in λ_{max}. For both solvents at 90% concentration, the far-UV CD spectrum indicate an increase in the ordered secondary structural elements (random → α-helix) and/or conformational transition (β-sheet → α-helix). This probably occurs in the first domain because the second domain is more stable in consequence of the larger size of its hydrophobic core [22]. As a result of the conformational rearrangement, the Trp residue located in the β-sheet region in the native enzyme may become the component of a newly formed α-helix. In an α-helix, the side-chains of the amino acids are more exposed to the solvent-polarity effects than in the β-sheet. The continuous increase in I_{max} and the blue shift of λ_{max} reflect progressive exposure of the Trp residues with increasing ACN or ethanol concentration. Comparison of the near-UV CD spectra at 90% shows that, in spite of the apparently insignificant difference in the secondary structures, the enzyme in ACN is more susceptible for tertiary structural change, particularly around the disulfide groups (Fig. 6B). This may have consequences on the catalytic activity of papain. An important part of the catalytic machinery of papain is the oxyanion hole located at the N-terminal end of the α-helix formed by residues 24–42. Within this region, the amide group of the only free cysteine, Cys25, contributes to one of the hydrogen bonds in the oxyanion hole [25]. Of the three disulfide bridges, Cys22–Cys63 is also located near the active site. The considerable loss in catalytic activity of papain at high ACN concentrations as compared with that observed in ethanol

can be explained by the profound alteration in the tertiary structure around the oxyanion hole.

4.2. Structural and catalytic behavior of papain in 1,4-dioxane and tetrahydrofuran

In the presence of these two solvents, the enzyme exhibits quite different catalytic activities: whereas in 90% THF it lost its activity completely, in 1,4-dioxane about 80% of the activity was preserved. The gradual loss in tertiary structure upon increase of the concentration of 1,4-dioxane and THF, in parallel with increasing I_{max} values and the red shift of λ_{max}, indicates that the enzyme structure becomes looser. At a solvent concentration of 90%, the tertiary structure is almost absent, but I_{max} decreases (in the case of THF, it drops to a value comparable to that observed in the native state) with a concomitant blue shift in λ_{max}. In general, unfolding of the proteins leads to an increased I_{max} and red-shifted λ_{max} values [26]. Hence, we can only speculate that the decreased I_{max} and the blue shift observed at 90% 1,4-dioxane and THF is (i) a consequence of a rearrangement of the protein structure resulting in a more compact structure different from that in the native state, or (ii) 90% 1,4-dioxane and THF comprise a more apolar environment for the solvent-exposed Trp residues in the unfolded enzyme as compared with that in the interior of the folded protein. This is supported by the blue shift of λ_{max} for the *N*-Ac-L-Trp-ethyl ester with increasing apolarity of the environment. As regards the results of our CD and fluorescence measurements, the highly different catalytic activities of papain observed in 1,4-dioxane and THF cannot simply be explained on the basis of tertiary structural change.

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