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UNFOLDING MECHANISM AND STABILITY OF IMMOBILIZED PAPAIN

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Abstract

The thermal stability of papain in free solution or immobilized on CPC-silica has been investigated by DSC. At neutral *pH*, in both conditions, the protein undergoes a thermal transition which corresponds to the sum of two transitions associated with the unfolding of the two domains of the protein. At low *pH*, in the case of immobilized papain, only one transition is observed.

Keywords: enzymes, microcalorimetry, papain, protein immobilization, protein melting domains

Introduction

Enzymes immobilized on solid supports are of potential interest as biocatalysts for processes at an industrial level. In order for this potentiality to be fully expressed, it is necessary to investigate the effects of immobilization on the properties of the enzyme not only at a practical technological level, but also from biochemical and molecular aspects. Recent studies of the mechanism of thermal stabilization and the unfolding pathway of model proteins (such as ribonuclease and chymotrypsin) have shed some light on the effects of immobilization [1, 2]. The present work continues the series of physico-chemical studies on the characterization of immobilized proteins in order to gain information of value for the best practical exploitation and performance of enzymes as biocatalysts.

Papain has long been used as a large-scale industrial enzyme [3]. Its applications (some of them patented since the beginning of the century) have ranged from beer chill-proofing (unspecific proteolytic treatment of haze) to medical care (digestive aid and anti-eczema drug) [4]. The stability to heat denaturation was early recognized: papain has been widely used to tenderize meat because its action continues during the early stages of cooking. In many applications for large-scale processes, an immobilized form of the enzyme may be a necessary requirement. For instance, papain was one of the first proteolytic enzymes immobilized on an inorganic support [5]. As a consequence, its activity was further stabilized against thermal inactivation. Although papain has often been

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used as a model protein to study immobilization techniques [6, 7], biochemical characterization of the immobilized macromolecule has been lacking.

The thermally-induced unfolding process of the free enzyme is characterized by a thermodynamic mechanism which is not consistent with the two-state hypothesis [8]. The protein behaves as an ensemble of two thermodynamically independent parts. Actually, X-ray reflection data have shown that the molecule is composed of two structurally well-defined lobes, separated by a deep cleft containing the active site [9].

In this report, the thermal denaturation and unfolding pathway were studied in detail. Papain was immobilized on aminoalkylated silica beads through a covalent link. The thermodynamic properties of the protein and its thermal stability were studied by DSC, in order to assess the changes with respect to the free enzyme. It was found that the mechanism of thermal unfolding after immobilization is indeed drastically altered as a function of the ionization state of the macromolecule.

Materials and methods

Dithiotreitol (DTT) and papain from *Carica papaya* (P 4762) were from Sigma. Controlled Pore Ceramics (CPC Silica Carrier, average pore size 375, a 3-aminotriethoxysilane derivative) was from Fluka.

Papain activation

Free-papain activation was carried out in the presence of 1 *mM* DTT+ 50 *mM* phosphate buffer, *pH* 7.2, for 2 h. Immobilized papain was activated in 1 *M* phosphate buffer, *pH* 7.8, +2 *mM* DTT for 2 h. The final composition of all the buffers included 1 *mM* DTT to prevent oxidation. The change in the sample *pH* was obtained by dialysis.

Immobilization

The immobilization procedure was similar to those reported previously [1, 2]. To a suspension of CPC aminopropyl derivative (10 g) in 1 *M* phosphate buffer, *pH* 7.8, 150 ml of 13% glutaraldehyde was added. After 30 min at 4°C, the beads were washed several times and added to 25 ml of papain solution (10 mg ml^{-1}), dialysed 3 times against the same buffer. The suspension was gently shaken for 12 h at 4°C and then thoroughly washed with 50 *mM* phosphate buffer, *pH* 7.2. The amount of bound protein was calculated by measuring the difference in absorbance (ε_{280} = 25 M^{-1} cm^{-1}) of the supernatant before and after immobilization and the absorbance of the first three washings. The immobilized protein was stored frozen after removal of the excess of water by filtration. Before use, the sample was washed several times with buffer of

given composition and *pH*. The biological activities of the samples were verified with BAEE (α-N-benzoyl-l-arginine ethyl ester).

Calorimetry

DSC experiments were performed with an MC-1 DSC Microcalorimeter (Microcal. Inc., Northampton, MA, USA) equipped with stainless cells (0.7 ml) for solid samples. The reference cell was filled with a suitable amount of untreated CPC (without protein) and buffer, in order to compensate the change in heat capacity of the sample due to the presence of the silica beads. The scan rate was 48°C h^{-1}. The concentrations of the free enzyme solutions were 1–2 mg ml^{-1}. The unfolding transitions were analysed according to the mathematical treatment of Freire and Biltonen [10, 11], based on the two-state hypothesis. The experimental curves were fitted with the program ORIGIN (Microcal. Inc., MA, USA). From the mathematical analysis, the thermodynamic parameters associated with the unfolding process, i.e. the enthalpy change, ΔH, the middle point transition temperature, T_m, and the ratio of the van't Hoff and calorimetric enthalpy changes, C_u, defined as cooperative unit, were calculated.

Results and discussion

Free papain in aqueous solution undergoes a cooperative unfolding transition as a function of temperature, centered at around 86°C at *pH* 7.2. The relatively high temperature of unfolding suggests a marked stability to heat denaturation. The fitting of the experimental DSC curves reveals that the overall transition cannot be approximated by a single process according to the two-state analysis (C_u different from 1). This confirms the results reported in the literature on the unfolding of the iodoacetamide-inactivated papain derivative [8]. In order to obtain the best fitting of the experimental curve, it was necessary to assume the presence of two consecutive transitions. Besides the calorimetric enthalpy change, ΔH_c, obtained by integration of the area beneath the experimental curve, the van't Hoff enthalpy change, ΔH_v, was also obtained from the temperature dependence of the equilibrium constant. The ratio $\Delta H_{vH}/\Delta H_c$, C_u, is a parameter usually considered a valid criterion for the establishment of two-state behaviour [12]. The thermodynamic parameters associated with each transition as a function of *pH* are listed in Table 1. The two consecutive transitions seem to occur independently because C_u is close to 1 for both of them. At very acidic *pH*, this is no longer true, and a significant departure from the theoretical value of $C_u = 1$ is observed. The overall ΔH (658 kJ mol^{-1}) and C_u (1.78) values are in reasonable agreement with those reported in the literature for papain unfolding at acidic *pH* [8].

Table 1 Thermodynamic parameters of thermal unfolding of papain in solution

pH	T_{m1} / °C	ΔH_{1c} / kJ mol^{-1}	ΔH_{1vh} / kJ mol^{-1}	C_{u1}	T_{m2} / °C	ΔH_{2c} / kJ mol^{-1}	ΔH_{2vh} / kJ mol^{-1}	C_{u2}	ΔH_{tot} / kJ mol^{-1}
7.2	84.3	369.0	349.4	1.06	90.9	447.7	440.6	1.02	816.7
5.0	87.3	363.2	344.3	1.05	91.1	462.8	462.3	1.00	825.9
3.4	81.3	260.2	359.0	0.72	88.1	398.3	481.6	0.83	658.6

Average of 2–3 experiments. Errors: $T_m = \pm 0.3$; $\Delta H = \pm 3\%$

pH 7.2 in 50 *mM* Phosphate buffer; *pH* 5.0, 3.4 in 50 *mM* Acetate buffer

The presence of two independent processes may be related to the bilobal structure of the protein molecule. X-ray diffraction analysis [9] indicates that the papain molecule consists of two lobes well separated by a deep cleft. Many other enzymes (such as ribonuclease, lysozyme and chymotrypsin) have similar composite structures, but they unfold as a single cooperative unit and exhibit overall two-state behaviour. This is not the case for papain. The two structurally well-defined parts of the molecule may correspond to two thermodynamically independent (or loosely coupled) substructures or domains. The two parts may also differ in intrinsic stability, as the analysis of the structure seems to indicate. For instance, the $-NH_2$-terminal domain folds around a compact hydrophobic core, and has a higher content of secondary structure, but includes a lower number of salt bridges and only one (out of three) disulfide bonds. Conversely, the –COOH– terminal domain displays a more irregular structure and contains less hydrophobic aminoacid residues. It should be noted that, apart from four short α-helical segments and one short β-structure (the total amount of secondary structure is 20%), the conformation of the rest of the protein is irregular. Nevertheless, the protein thermostability is noteworthy, at least with respect to that of other more structured proteins.

The chemical coupling between papain and the support was achieved under experimental conditions where only 1–2 bonds are usually formed, as observed with other proteins [1, 2]. Glutaraldehyde was used as cross-linking reagent because it readily reacts with the primary amino groups in the unprotonated form. Under these conditions, the terminal $-NH_2$ group is the most favoured group for the coupling reaction because in papain (as in many other enzymes) it has a *pK* significantly lower than that of the other primary $\varepsilon-NH_2$ [9]. As a consequence of the chemical linkage with the support, after immobilization the thermody-

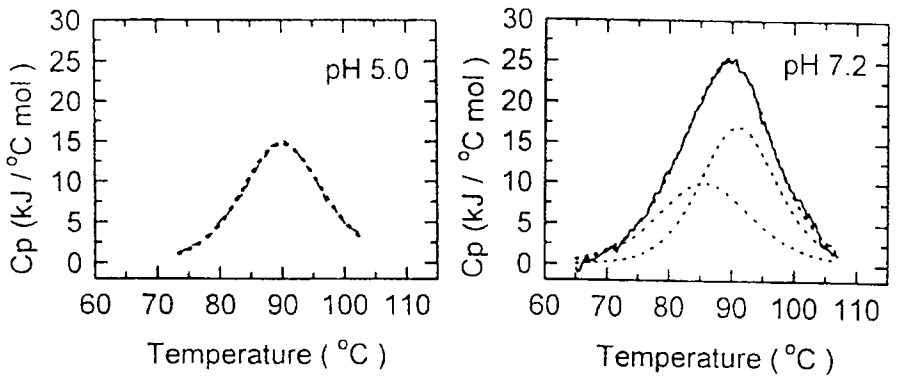

Fig. 1 Temperature dependence of the excess specific heat capacity of papain immobilized on CPC-silica: 50 *mM* Na acetate buffer, *pH* 5.0 and 50 *mM* Na phosphate buffer, *pH* 7.2. Dotted lines: best fits according independent two state transition of the experimental curves

namic behaviour of papain is significantly changed. The protein still undergoes a cooperative transition, which does not exhibit overall two-state behaviour. However, whereas the unfolding process has to be approximated by two independent and overlapping transitions at *pH* 5.5 (Fig. 1), below *pH* 5.5 one of the two transitions disappears. Only the higher-temperature pro-cess still persists below *pH* 5.5 (Fig. 1).

The thermodynamic parameters associated with the transitions are listed as a function of *pH* in Table 2. It can be seen that both transitions strictly obey two-state behaviour, except at very low *pH*. The two protein domains seem to be independent, since they unfold separately, suggesting that domain decoupling still persists in the immobilized molecule. The disappearance of the low-temperature process indicates that the corresponding domain is already in the unfolded form below *pH* 5.5. No alteration in the behaviour of the remaining peak is observed as a function of *pH*, since T_{m2} (Fig. 2A) also varies smoothly as a function of *pH* when the first transition disappears.

Why is this drastic structural change observed in the immobilized protein? The covalent links with the support, and also the interactions with the silica surface, perturb the conformation of the protein molecule, inducing changes which not only alter the interdomain interactions, but also have an effect (either positive or negative) on the stability of each domain. The surface of the silica beads is slightly negatively charged due to the presence of weakly acidic siolic groups. On lowering of the *pH* of the medium, the changes induced in the ionization states of both papain and the support contribute to alter the protein stability. Similar effects as a function of *pH* have been detected with other immobilized enzymes on the same inorganic carrier [1, 2], especially when the corresponding free proteins undergo conformational changes as a function of *pH*. It should be noted that, similarly in the case of papain, studies of the *pH* dependence of the fluorescence emission, characterized by the existence of a well-defined histidine-tryptophan complex, revealed marked changes between *pH* 5 and *pH* 8.5 [13]. Moreover, the free enzyme tends to aggregate below *pH* 6 [14]. Finally, it is interesting to note that the bell-shaped *pH* dependence of the esterolytic activity of papain has a maximum at around *pH* 5–5.5 [6].

As shown in Table 2, T_{m2} is similar for the free and the immobilized enzyme forms being only marginally higher for the latter near neutral *pH*. On the other hand, larger differences are observed for the enthalpy changes. Immobilized papain has an overall ΔH_c and also single-process ΔH_c values significantly lower than those of the free enzyme. The reasons for such a discrepancy, which were observed for the α-chymotrypsin [1], but not for ribonuclease A [2], are not clear. One possible explanation is that a fraction of the bound protein molecules are in the unfolded state. However, this may not be the case because the values of ΔH_v (which is not sensitive to the protein concentration) are lower accordingly. Another possibility is that the initial (or final state) is thermodynamically

Table 2 Thermodynamic parameters of thermal unfolding of papain immobilized on CPC-silica

pH	T_{m1} / °C	ΔH_{1c} / kJ mol^{-1}	ΔH_{1vh} / kJ mol^{-1}	C_{u1}	T_{m2} / °C	ΔH_{2c} / kJ mol^{-1}	ΔH_{2vh} / kJ mol^{-1}	C_{u2}	ΔH_{tot} / kJ mol^{-1}
7.2	85.6	212.1	214.6	1.01	91.3	269.4	274.1	1.02	481.6
6.5	86.8	210.0	225.5	1.07	93.9	270.3	286.2	1.06	480.3
6.0	87.5	220.1	227.6	1.03	94.3	269.9	277.0	1.03	489.9
5.5	84.1	216.7	219.7	1.01	95.2	263.6	279.5	1.06	480.3
5.0					90.2	259.0	249.4	0.96	259.0
4.5					89.1	255.2	253.1	0.99	255.2
4.0					89.0	245.6	244.3	0.99	245.6
3.6					89.4	236.4	240.6	1.02	236.4
3.5					87.6	228.0	252.3	1.11	228.0
3.0					81.2	202.1	248.9	1.23	202.1
2.5					75.2	153.6	256.5	1.67	153.6
3.0*					60.3	190.4	199.6	1.05	190.4

* RESCAN

Errors: $T_m = \pm 0.3$°C; $\Delta H = \pm 3.5\%$.

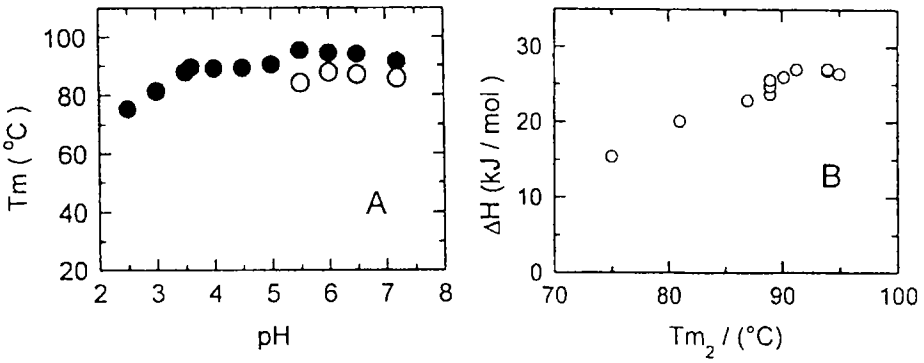

Fig. 2 A) Dependence on *pH* of the melting temperatures of molecular domains in papain immobilized on CPC-silica. (filled circles): second domain; (open circles): first domain; B) ΔH of unfolding of second domain *vs.* T_m

(and structurally) different in the case of the immobilized enzyme, probably because of some conformational rearrangement of the molecule after immobilization. All three proteins studied (α-chymotrypsin, ribonuclease A and papain) have a *pH* above 9. Therefore, the degree of recovery of the overall enthalpy changes does not seem to be correlated with the ionization state of the protein. Hydrophobic interactions with the glass surface may be as relevant as electrostatic and hydrophilic interactions on determining the protein conformations after immobilization.

The overall unfolding transition is not reversible near neutral *pH*, in the sense that following a temperature scan and successive cooling no evidence of an unfolding transition was observed after a second temperature scan. Conversely, at low *pH*, an almost complete recovery of the calorimetric ΔH was observed even though the T_m of the second scan is shifted to a significantly lower temperature probably induced by the presence of the uncharged surface of the support. (Table 2, *pH* 3).

It should be noted that the unfolding transitions of several proteins do conform to equilibrium thermodynamic behaviour in spite of the irreversibility after a second temperature scan. This may be considered a too strict criterion of reversibility, as convincingly discussed by Sturtevant [15–17]. In fact, during the first heating, protein unfolding may approach true thermodynamic equilibrium behaviour for most part of the transition, although at temperatures at the end or well above complete unfolding this may be true anymore.

In Fig. 2B, the enthalpy change associated with the unfolding of the remaining peak, ΔH_2, is plotted as a function of the corresponding transition temperature, T_{m2}. The plot is approximately linear, with a slope (the apparent heat capacity change associated with the unfolding, ΔC_p) equal to 6.3 kJ K^{-1} mol^{-1}. This value is half of the ΔC_p calculated for the whole free papain

(13.7 kJ K^{-1} mol^{-1} [8]). This suggests that contributions to the heat capacity change are similar for the two protein domains, which is surprising in view of the different compositions and structures of the two protein lobes.

In conclusion, papain is a fairly heat-resistant protein. After immobilization on silica beads by covalent attachment, the thermal stability is only marginally improved. The mechanism of unfolding is drastically altered: at low *pH* (below *pH* 5.5), one of the two protein domains seems to be in the unstructured form. This may give some hint as to how immobilized catalysts should be handled in order to exploit them as biocatalysts for relevant chemical processes.

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