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ORIGINAL ARTICLE

Cysteine enhances activity and stability of immobilized papain

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Abstract Immobilization of papain on Sepharose 6B in the presence of different concentrations of cysteine affected the enzyme activity depending on cysteine concentration. The maximum specific activity was observed when papain was immobilized with 200 mM cysteine. The immobilization process brought significant enhancement of stability to temperature and extreme pH values with respect to free papain. After immobilization, the optimum temperature of papain activity increased by 20°C (from 60 to 80°C) and its optimum pH activity shifted from 6.5 to 8.0. Catalytic efficiency (k_{cat}/K_m) and specific activity of the immobilized enzyme do not significantly change after immobilization. The temperature profile of this form of immobilized papain showed a broad range of activity compared with both free and immobilized form of papain in the absence of cysteine. This significant behavior in terms of activation energy is also discussed.

Keywords Papaine · Cysteine · Enzyme immobilization · Thermal stability

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Introduction

In recent years, wide attention has been directed toward the potentiality of immobilized enzymes (Bommarius and Riebel 2004; Tischer and Kasche 1999). Compared with their free forms, immobilized enzymes are generally more stable and easier to handle. In addition, the reaction product is not contaminated by the enzyme (especially useful in food and pharmaceutical industries) (Reshmi et al. 2006) and, in the case of protease, the rate of the autolysis process can be dramatically reduced on immobilization (Gianfreda and Scarfi 1991).

Papain, a thiol protease present in the latex of *Carica papaya*, exhibits broad proteolytic activity and is an enzyme of high research interest, having also a variety of industrial applications. In the food industry, papain is used to tenderize meat and related derivatives, to produce protein hydrolysate, to clarify juice and beer in the brewing industry, for cheese production in dairy industry, in baking industry and for the extraction of flavor and color compounds from plants. Papain can also be used in the forage industry to increase protein content and in recovering plant and animal protein to make health products (Monti et al. 2000; Rehm and Reed 1987; Sumantha1et al. 2006). The potential uses of papain include: synthesis of amino acids, esters and peptides; treatment of acute destructive lactation mastitis; treatment of red blood cells prior to use in antibody-dependent cell-mediated cytotoxicity assays with lymphocytes; in the enzyme inhibition-based biosensors for food safety and environmental monitoring (Afaq and Iqbal 2001; Shukor et al. 2006). The immobilization of papain is useful because of its great industrial applications and, in many instances, the immobilized form of the enzyme shows advantageous characteristics with respect to its soluble form.

It is now wildly accepted that the activity of papain is dependent on the establishment of a thiolate–imidazolium ion pair of Cys-25 and His-159 in the active site (Harrisonet al. 1997). Papain is activated by cysteine and it is required for complete activation. The –SH group of Cys-25 is highly reactive. Kinetic studies indicate that thiols may activate papain, in part by increasing the rate of decomposition of the enzyme–substrate complex, but the mechanism of activation is not completely clarified (Sanner and Pihl 1963).

One major problem in enzyme immobilization, particularly by covalent linkage, is the loss of enzyme activity, which is attributed to many factors involving enzyme, matrix, reagents and process conditions (Delanoy et al. 2005; Srinivasa Rao 2006). Several strategies have been employed to improve the catalytic activity of immobilized enzymes, including immobilization of the enzyme in the presence of saturating concentration of substrate, product or competitive inhibitors (Chaplin and Buke 1970), introduction of spacer arms between enzyme and support (Brown et al. 1970), use of oriented mode of binding to materials (Hipwell et al. 1974; Wilchek and Miron 2003), chemical post-immobilization techniques (Blanco and Guisan 1992; Rocha et al. 1998) and manipulating the structure of carrier materials for enzyme immobilization (Kim et al. 2006).

In recent years, papain has been immobilized onto various supports and through different methods (Axen and Ernback 1971; Sangeetha and Abraham 2006); these often resulted in the reduction of enzyme activity. In an attempt to improve the specific activity of the immobilized form of papain and to increase its catalytic efficiency, we performed the contemporary immobilization of the enzyme and its activator, cysteine, on CNBr-activated Sepharose 6B. The efficacy of this immobilization procedure was checked in terms of papain activity and stability, comparing the kinetic parameters with those referred to its soluble form.

Materials and methods

Chemicals

Sepharose 6B, cysteine and cyanogen bromide (CNBr) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were reagent grade and purchased from Merck (Darmstadt, Germany).

Activation of Sepharose and immobilization of papain

Sepharose 6B was activated with cyanogen bromide. The titration method of activation was carried out for activating Sepharose and papain coupled to the CNBr-activated Sepharose at a concentration of 5 mg/ml, according to the procedure described by (March et al. 1974). The ratio of gel/protein solution (v/v) was 1.5:1. The gel was treated with 2 M glycine or 2 M cysteine to block the remaining activated group on the matrix after immobilization. The immobilization process was also carried out in the presence of different concentrations of cysteine.

Evaluation of the cysteine linked to the matrix

The amount of the cysteine covalently linked to activated sepharose was quantified by iodometric titration, according to Bernkop-Schnurch et al. (2001). In brief, 500 µl of 1% starch solution (w/v) was added to 1 mL of gel, at a pH value previously adjusted to 2–3 by 1 M HCl. The sample was titrated with 1 mM iodine solution until a permanent light blue color appeared.

Determination of protease activity and protein concentration

Protease activity was determined at room temperature in 50 mM phosphate buffer, pH 7.5, containing 38 mM EDTA and 34 mM cysteine chloride, using casein as substrate, by the modified method of (Kembhavi et al. 1993). To 50 µl of enzyme solution diluted in 350 µl of buffer, 400 µl of 1% (w/v) aqueous solution of casein was added and the reaction mixture was incubated at room temperature for 10 min. The reaction was stopped by adding 800 µl of 10% trichloroacetic acid (TCA) solution. The mixture was incubated at room temperature for 30 min, centrifuged at 12,000*g* for 10 min and the absorbance of the supernatant measured at 280 nm. One unit of protease is defined as the amount of enzyme that hydrolyzes casein to produce equivalent absorbance to 1 µmol of tyrosine/min with tyrosine as standard. In the case of the immobilized papain, the reaction mixture was continuously stirred during the reaction.

Protein concentration was estimated by the Bradford method (Bradford 1968), using bovine serum albumin as standard.

Determination of optimum pH and temperature

The activity versus pH profiles of free and immobilized papain were graphed measuring the papain activity at room temperature, as reported above, in a mixed buffer containing 50 mM acetate, phosphate and glycine at different pH values. Similarly, the activity versus temperature profiles were graphed on the basis of the activity values measured at different temperatures in the range 30–90°C, in phosphate buffer of pH 7.5, as described.

Effect of pH and temperature on enzyme stability

To determine the thermal stability, free and immobilized papain were incubated at 80 and 90°C in 50 mM phosphate buffer, pH 7.5, for different intervals of time, then cooled on ice and the residual activity determined under the assay conditions. The stability to pH was checked after incubation of the enzyme in 50 mM of mixed buffer (pH 3 and 12) for different intervals of time at room temperature, then pH value was adjusted to 7.5 and the residual activity determined according to the assay conditions. Control measurements were carried out measuring the activity of the same enzyme solution kept on ice for the thermal stability and in the buffer at pH 7.5 for the pH stability experiments.

Determination of kinetic parameters

Catalytic activities of free and immobilized papain were investigated at different substrate concentrations under assay conditions. K_m, V_{max} and k_{cat} values were determined by Lineweaver–Burk plots.

Results and discussion

The efficiency of coupling immobilization using 200 mg of CNBr per milliliter of Sepharose 6B and 5 mg/mL of papain in 0.2 M NaHCO3 at pH 9.5 (1:1.5 ratio of gel/protein solution, v/v) was 7.3 mg of protein per ml of packed activated gel. The yields of activity and immobilization were about 53 and 76%, respectively.

Catalytic activity of the immobilized enzyme in the presence of cysteine

Cysteine or glycine were used to block the activated sites of the gel not occupied by enzyme. We verified that when cysteine was used, the specific activity of the immobilized papain was about 28% higher than that obtained when glycine was alternatively used. To verify this result, papain was immobilized in the presence of different concentrations of cysteine in the range 100–2,000 mM (referred to as co-immobilized enzyme). The resulting specific activities are shown in Fig. 1, where the notation 0 mM cysteine means that glycine alteratively to cysteine was used to block the gel sites (we called it immobilized enzyme). From the experimental data, it appeared that the maximum specific activity occurred when papain was co-immobilized with 200 mM cysteine. By iodometric assay, it was found that cysteine was linked (data not shown). The data suggest that the presence of cysteine induces conformational changes of papain in its more active form and the immobilization of the enzyme on the support induces the retention of this activated form.

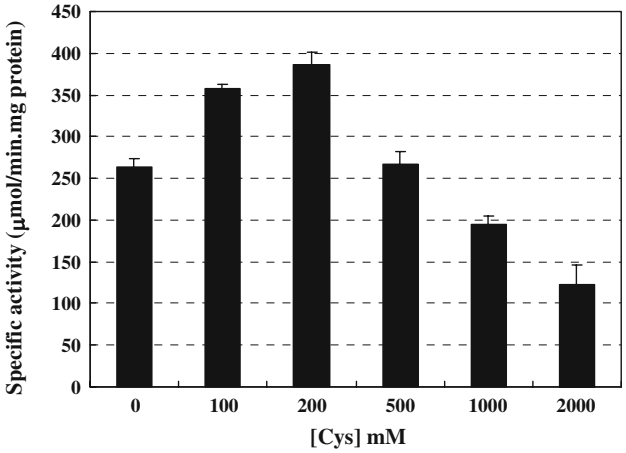

Fig. 1 Effect on the specific activity of immobilized papain of different concentrations of cysteine during immobilization

Effect of cysteine concentration on enzyme activity

The effects of cysteine concentration in the range 0–40 mM on the specific activity of free and both papain immobilized forms were compared. From Fig. 2 it appears that the maximum activity of the simple immobilized enzyme was found in the presence of 10 mM cysteine, while in the case of co-immobilized enzyme, the highest activity was with 5 mM cysteine. When cysteine was not present in the medium, the specific activity of enzyme co-immobilized with cysteine was higher than the free and immobilized enzyme. These results indicate that when enzyme is co-immobilized in the presence of 200 mM cysteine, its dependence on the free cysteine is reduced compared to both free and simple immobilized enzyme, so that in the absence of cysteine its specific activity is even higher than free enzyme.

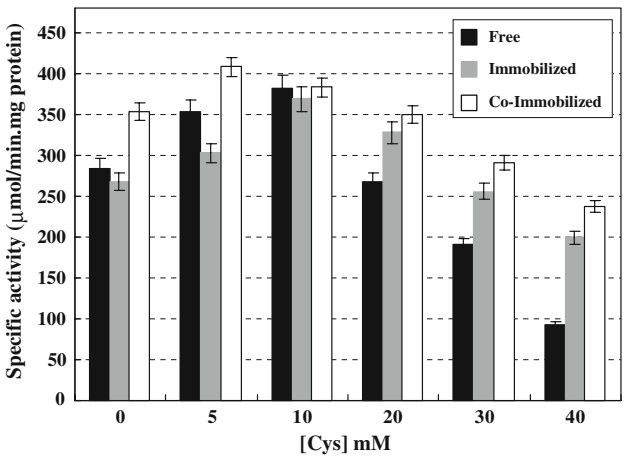

Fig. 2 Effect of different concentrations of free cysteine on the specific activity of free and immobilized forms of papain

Effect of immobilization on kinetic parameters

Papain and its immobilized forms revealed a Michaelis–Menten type of kinetics when hydrolyzed with casein. As shown in Table 1, K_m and k_{cat} values, calculated by the Lineweaver–Burk plots, do not undergo significant variation after immobilization, contrary to that reported in literature for most enzymes (Reshmi et al. 2006; Arslan et al. 2005; Cetinus and Öztop 2003; Sahmetlioglu et al. 2006).

Speculatively, a slight increase in K_m and significant decrease in k_{cat} of immobilized papain at room temperature compared with its free form may be observed. As known, the slight increase in K_m value may be due to diffusion limitations, steric hindrance of the active site and/or loss of enzyme flexibility necessary for substrate binding (Cetinus and Öztop 2003; Jiang et al. 2005), while the decrease in k_{cat} could be due to the restricted diffusion of substrate to the active site and the higher structural rigidity of immobilized enzyme.

This tendency appears inverted when papain is co-immobilized with 200 mM cysteine: K_m and k_{cat} values decreased and increased, respectively, compared with those referred to immobilized and free enzyme. The catalytic efficiency of this form of immobilized papain shows a lower decrease than simple immobilized enzyme, compared to the free form. Evidently, the role of cysteine appears confirmed in the catalytic process also when the enzyme is immobilized.

Effect of immobilization on temperature and pH profiles and activation energy

The influence of temperature and pH on the activity of free and immobilized enzymes is shown in Fig. 3. A significant shift to higher temperatures was observed on immobilization: in fact, maximum activity at 60°C for free papain and 80°C for two forms of immobilized enzyme were found. Co-immobilization of the enzyme in the presence of 200 mM cysteine showed no effect on optimum temperature of immobilized enzyme, but the co-immobilized form was more active at low temperatures compared to the immobilized enzyme. This behavior of the co-immobilized enzyme appears interesting considering that, in general, the immobilization causes a reduction in the activity of enzymes at low temperatures, such as immobilized esperase

Table 1 Kinetic parameters of free and immobilized forms of papain

Enzyme form	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})
Free papain	27×10^{-5}	0.62	4.4×10^2
Immobilized papain	17×10^{-5}	0.79	2.2×10^2
Co-immobilized papain	21×10^{-5}	0.57	3.7×10^2

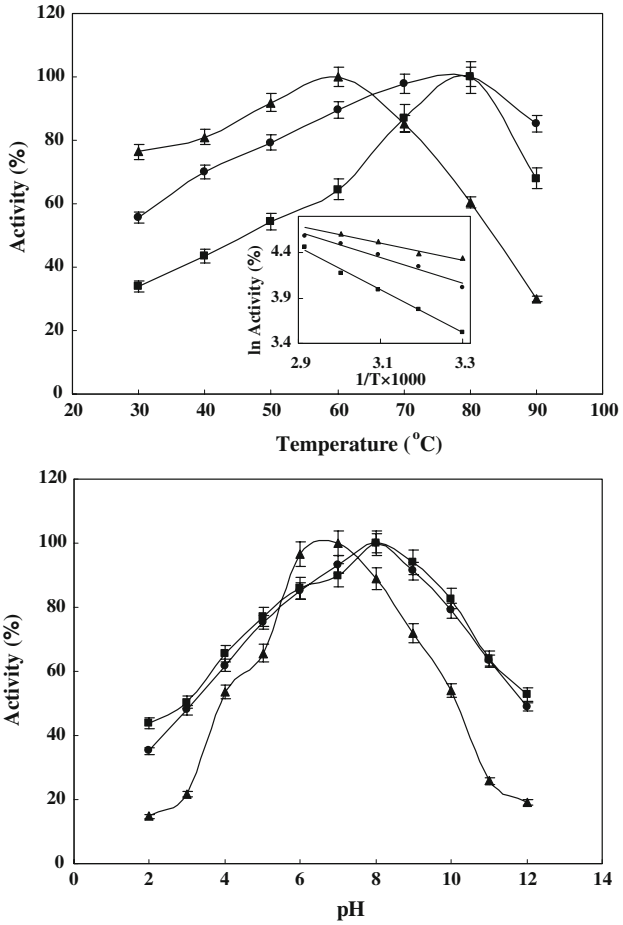

Fig. 3 Effect of temperature and pH on the activity of free (*filled triangles*), immobilized (*filled squares*), and co-immobilized enzyme in the presence of 200 mM cysteine during immobilization (*filled circles*). In the *inset* are the respective Arrhenius plots

on Eudragit S-100 (Silva et al. 2006), immobilized pepsin on chitosan beads (Altun and Cetinus 2007) and immobilized catalase on crosslinked chitosan beads (Cetinus and Öztop 2003). The free form of enzyme shows high activity at low temperature, but significantly decreases at temperatures higher than 70°C. On other hand, the immobilized enzyme shows lower activity at low temperatures and higher activities at high temperatures, while the co-immobilized enzyme in the presence of 200 mM cysteine shows higher activity with respect to the simply immobilized enzyme at low temperatures and free enzyme at high temperatures. The enhancement of the catalytic activity at high temperature, which could be helpful for basic investigations and technical applications, presumably occurs as a consequence of the prevention of autolysis, facilitated at high temperature, due to the linking of the enzyme to the support.

The Arrhenius plots (inset of Fig. 3) were graphed utilizing the activity values in the temperature range of 30–60°C and 30–70°C for papain and its immobilized forms, respectively. The activation energies of the enzymatic

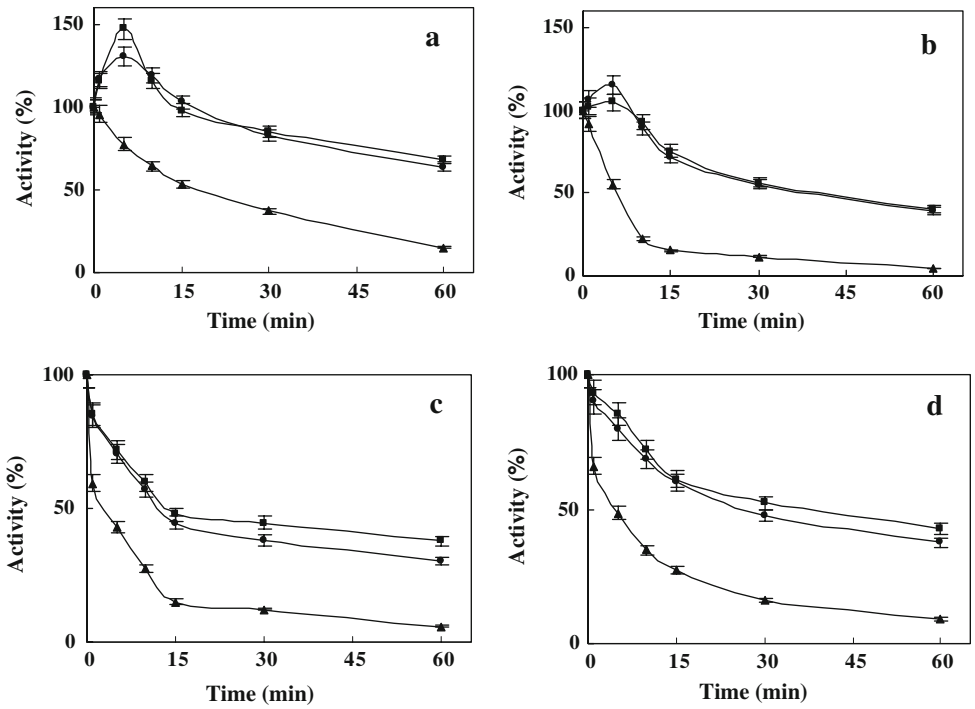

Fig. 4 Irreversible thermoinactivation at 80°C (**a**) and 90°C (**b**) and stability at pH 3.0 (**c**) and 12 (**d**) for free (*filled triangles*), immobilized (*filled squares*) and co-immobilized papain in the presence of 200 mM cysteine during immobilization (*filled circles*)

reaction, calculated by the slopes of the plots, decreased from 4.69 to 2.84 kcal mol^{-1} K^{-1} when cysteine was present during immobilization. The activation energy of free papain, 1.87 kcal mol^{-1} K^{-1} for the hydrolysis of casein, was close to that found in the case of co-immobilized papain. These results suggest a decrease in sensitivity to temperature and an increase in conformational rigidity of the co-immobilized enzyme compared with immobilized enzyme and its free form, respectively.

The pH value of maximum activity of papain was shifted from 6.5 to 8.0 on immobilization. The pH profiles of the two forms of immobilized papain were coincident and showed a broad pH activity profile compared with that of free papain. The immobilized forms of enzyme showed higher activity both in acidic and basic pH ranges, when compared to the free form. The shift of maximum activity of covalently coupled papain toward alkaline pH values, reported by others (Srinivasa Rao et al. 2006; Sangeetha and Abraham 2006), could be due to the increase in H^+ ion concentration on the microenvironment of the immobilized enzyme and/or related mainly to the formation of an enzyme support complex characterized by a suitable open conformation with little restriction to the access of substrates (Srinivasa Rao et al. 2006; Jiang et al. 2005).

Effect of immobilization on thermostability and stability at extreme pHs

Irreversible thermoinactivation of free and immobilized forms of papain was verified at 80 and 90°C. As shown in Fig. 4a, b both immobilized forms of papain exhibited a marked increase in thermostability and stability with time, compared with the free form. Besides, two immobilized forms of enzyme showed approximately the same thermostability. At 90°C, the half life of immobilized enzyme was 40 min, while it was 6 min in the case of the free form. After 60 min of incubation at 90°C, immobilized enzymes retained 45% of their original activity, while negligible activity was observed in the case of the free enzyme.

Immobilization of papain on Sepharose 6B could cause an increase in conformational rigidity of protein structure and a limited susceptibility to drastic conformational changes (Jiang et al. 2005). On the other hand, the fixation of enzyme on the support can lead to a lower degree of autolysis, consequently increasing the stability of the enzyme. The improvement of thermal stability of papain after immobilization has been reported (Afaq and Iqbal 2001; Srinivasa Rao et al. 2006; Sangeetha and Abraham 2006), but improving thermal stability achieved in this work for papain was not observed.

The stability of the free and the two forms of immobilized papain was measured at two extreme pH, 3.0 and 12. As shown in Fig. 4c, d, the immobilized enzymes were more stable compared with the free enzyme at both pH values. In fact, after 60 min of incubation, the immobilized papains retained about 50% of residual activity, while, under the same conditions, a nearly complete inactivation of free papain was observed. As previously reported (Sangeetha and Abraham 2006; Lei et al. 2004), this indicates that immobilization appreciably enhanced the stability of papain both

in the acidic and basic pHs. Like irreversible thermoinactivation, the presence of 200 mM cysteine together with papain during immobilization does not influence the resistance of immobilized enzyme to extreme pHs.

In conclusion, an immobilized enzyme with a higher stability and a broad range of temperature profile, such as co-immobilized papain with 200 mM cysteine, could be a suitable candidate for industrial applications. This result is particularly important considering the fact that one major problem in enzyme immobilization is the loss of enzymatic activity and catalytic efficiency.

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