Computer-aided design of bromelain and papain covalent immobilization

Diseño asistido por computadora de la inmovilización covalente de bromelina y papaína

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Abstract

Enzymes as immobilized derivatives have been widely used in Food, Agrochemical, Pharmaceutical and Biotechnological industries. Protein immobilization is probably the most used technology to improve the operational stability of these molecules. Bromelain (*Ananas comosus*) and papain (*Carica papaya*) are cystein proteases extensively used as immobilized biocatalyst with several applications in therapeutics, racemic mixtures resolution, affinity chromatography and others industrial scenarios. The aim of this work was to optimize the covalent immobilization of bromelain and papain via rational design of immobilized derivatives strategy (RDID) and *RDID*$_{1.0}$ program. Were determined the maximum protein quantity to immobilize, the optimum immobilization pH (in terms of functional activity retention), was predicted the most probable configuration of the immobilized derivative and the probabilities of multipoint covalent attachment. As support material was used Glyoxyl-Sepharose CL 4B. The accuracy of *RDID*$_{1.0}$ program´s prediction was demonstrated comparing with experimental results. Bromelain and papain immobilized derivatives showed desired characteristics for industrial biocatalysis, such as: elevate pH stability retaining 95% and 100% residual activity at pH 7.0 and 8.0, for bromelain and papain, respectively; high thermal stability at 30 °C retaining 90% residual activity for both immobilized enzymes; a catalytic configuration bonded by immobilization at optimal pH; and the ligand load achieve ensure the minimization of diffusional restrictions.

Key words: bromelain, covalent immobilization, immobilized derivatives, papain, rational design.

Resumen

Las enzimas inmovilizadas han sido ampliamente utilizadas en las industrias Alimentaria, Agroquímica, Farmacéutica y Biotecnológica. La inmovilización de proteínas es, probablemente, la tecnología más empleada para elevar la estabilidad operacional de estas moléculas. La bromelina (*Ananas comosus*) y la papaína (*Carica papaya*) son cisteín proteasas extensamente usadas como biocatalizadores inmovilizados con disímiles aplicaciones en la terapéutica, resolución de mezclas racémicas, cromatografía de afinidad, entre otros escenarios industriales. El objetivo del presente trabajo fue optimizar la inmovilización covalente de las enzimas bromelina y papaína a través de la estrategia de diseño racional de derivados inmovilizados (RDID) y el programa *RDID*$_{1.0}$. Se predijo la cantidad máxima de proteína a inmovilizar, el pH óptimo de inmo-

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vilización (en términos de retención de la actividad funcional), la configuración más probable del derivado inmovilizado y la probabilidad de enlazamiento covalente multipuntual. Como soporte de inmovilización de empleó Glioxil-Sepharose CL 4B. La precisión de las predicciones llevadas a cabo con el programa *RDID$_{1.0}$* fue validada comparando con los resultados experimentales obtenidos. Los derivados inmovilizados de bromelina y papaína mostraron características deseadas para la biocatálisis a nivel industrial, tales como: elevada estabilidad al pH reteniendo el 95% y 100% de actividad residual a pH 7.0 y 8.0, para la bromelina y la papaína, respectivamente; una elevada estabilidad térmica con la retención del 90% de actividad residual a 30 °C para ambas enzimas; al pH de inmovilización óptimo la configuración obtenida es catalíticamente competente; y la carga de ligando alcanzada asegura la disminución de las restricciones difusionales.

Palabras clave: bromelina, derivados inmovilizados, diseño racional, inmovilización covalente, papaína.

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Introduction

Enzymes are widely exploited in biocatalysis to produce a broad spectrum of fine chemicals, pharmaceuticals and their building blocks, as well as commodity and agrochemicals (Liu *et al.*, 2004). The weak nature of enzyme limits its application at industrial level, beings one of the greatest problems in the biotechnological context (Soetaert and Vandamme, 2006; Mateo *et al.*, 2007; Iyer and Ananthanarayan, 2008; Hernández and Fernández-Lafuente, 2011).

The employment of enzymes in large-scale processes often encounters the problem of inactivation of enzyme. For example, at elevate temperatures enzyme undergoes partial unfolding due to the destruction of non-covalent interactions, and drastic pH variations affects the state of ionization of catalytical aminoacids. For technological and economical reasons, industrial processes have to be done by using immobilized enzyme derivatives. In particular, immobilization by covalent coupling retains very high enzyme activity over wide ranges of pH and temperature (Kunamneni *et al.*, 2008). The nature of the support and the methodology employed for immobilization results in diverse catalytic properties, stability, costs and handling properties. The alternative of using immobilized enzymes is advantageous because the enzyme could be recovered and usefully reused (Illanes, 2008; Hanefeld *et al.*, 2009).

Nowadays, affinity chromatography appears as one of the most efficient methods for protein separation and purification (Hage, 1999; Oh-Ishi *et al.*, 2002). The technique offers high selectivity, resolution, and usually high capacity for the molecule of interest; hence had been used on separation of natural or genetically synthesized proteins and peptides from complex mixtures (Polanowski *et al.*, 2003). Affinity chromatography has demonstrated its effectiveness on separation of proteolytic enzymes (employing substrates, substrates analogs, or proteases inhibitors as ligands) and its inhibitors (immobilized proteases as affinity matrix) (Delfin *et al.*, 1996; Anvar and Saleemuddin, 2002). Based on their importance in health and disease, protease inhibitors have already been developed into blockbuster drugs and diagnostics, many others are in clinical trials, and some proteases are themselves being trialed as vaccines or diagnostics (Abbenante and Fairlie, 2005). Hence, the design, synthesis and purification of protease inhibitors may result in potential therapeutic agents (Hugli, 1996; Leung *et al.*, 2000).

Recently, the lack of guidelines that could manage the selection of the best conditions for immobilization opens new opportunities for effective strategies related to the rational design of immobilized derivatives (RDID). New mathematical algorithms and bioinformatics tools (implemented into the program *RDID$_{1.0}$*) should be are harmonically combined for designing an optimal immobilization process (Torres-Salas *et al.*, 2011; del Monte-Martínez and Cutiño-Avila, 2012). In this work, *RDID$_{1.0}$* program was employed for the optimization of the covalent immobilization of the cystein proteases bromelain and papain in Glyoxyl-Sepharose CL 4B support for synthesizing optimal affinity chromatography matrix for protease inhibitors purification. The accuracy of *RDID$_{1.0}$* program prediction was evaluated with the obtained experimental results.

Materials and methods

Computational Methods: Coordinate files were obtained from Worldwide Protein Data Bank (PDB) (http://www.rcsb.org/pdb). PROPKA was used to predict the pKa values of protein ionizable groups (http://nova.colombo58.unimi.it/propka.htm). The Universal Protein Resource Knowledgebase (UniProtKB) was used to obtain functional information of proteins (http://www.uniprot.org/). *RDID$_{1.0}$* program has been proposed recently for optimization the immobilization processes and allows to calculate the following parameters: ligand interacting groups reactivity (*LIGR*e), reactivity index (*RI*), protein diameter (*d*), theoretical maximum protein quantity (*tMQ*), operational effectiveness coefficient (OEC), and the estimated maximum protein quantity (*eMQ*) (del Monte-Martinez and Cutiño-Avila, 2012).

Materials: Bromelain (EC 3.4.22.32, PDB ID: 1W0Q) was gently donated by Bioplants Center, Ciego de Ávila, Cuba. Papain (EC 3.4.22.2, PDB ID: 9PAP) and bovine serum albumin (BSA) were provided by Fluka Chemie AG, Switzerland. The reactive: Coomassie Blue G-250 (Brilliant Blue G), $NaHCO_3$, HCl, NaOH, NaH_2PO_4, $C_2H_3NaO_2$, NaCl, 1,2-epoxypropan-3-ol,

$NaBH_4$, and $NaIO_4$, were obtained from Sigma-Aldrich Co, USA. Sepharose CL 4B was obtained from GE Healthcare, USA.

Protein immobilization: The initial protein quantity taken as a reference was the estimated maximum quantity (*eMQ*), calculated with *RDID1.0* program. Glyoxyl-Sepharose CL 4B support, prepared according to Guisán *et al.* (1988), was suspended in 100mM $Na_2B_4O_7$100mM pH 8.0 or 100mM $NaHCO_3$ at pH 9.0 or pH 10.0; once the protein was added the final ratio support: immobilization solution was 1:10 (v:v). Then, the system was kept at 4°C under mechanical stirring during 2 hours. Finally, the appropriate amount of $NaBH_4$ was added to reduce the remaining aldehyde groups of the support to inert hydroxyl groups. In this way, the unstable Schiff´s bases formed between the support aldehyde groups and protein amine groups were transformed into more stable covalent bonds. All experiments were performed at least by triplicate. The control parameter of the immobilization process calculated was the Differential Immobilization Grade (*diff. IG*) according to del Monte *et al.,* (2002).

pH stability of soluble and immobilized enzymes: The pH stability was determined by preserving soluble and immobilized enzymes in various buffers: 50 mM sodium acetate buffer (CH_3COONa) pH 4-5, 50 mM Na_2HPO_4 pH 6-7, 50 mM Tris-HCl pH 8, 50 mM sodium bicarbonate buffer ($NaHCO_3$) pH 9-11. The flasks were sealed and incubated during 2 h. Finally, the residual activity was measured under standard conditions.

Thermal stability of soluble and immobilized enzymes: The thermal stability was determined by preserving at various temperatures (range 10-60 °C) sealed flasks with soluble and immobilized enzymes for 2 h in the standard buffer (50 mM Na_2HPO_4 pH 7.0). The residual activity was measured under standard conditions.

Protein concentration: Was determined by Coomassie Blue G-250 Assay using bovine serum albumin as standard protein (Bradford, 1976).

***Cystein protease assay*:** Enzymatic activities of bromelain and papain were determined according to Mole and Horton (1973). The synthetic compound N-Benzoyl-DL-Arginine-p-Nitroanilide (BAPA) was employed as a substrate. This compound was dissolved initially in 115mmol/L of dimethyl-sulfoxide (Berger and Schechter, 1970). The reaction mixture contained: 30µL of enzymatic extract taken for the immobilization, 10 µL of 4mmol/L BAPA in 130µL of activity buffer (0.3 mol/L of sodium acetate, 2 mmol/L of EDTA, 20 mmol/L of cystein-HCl at pH 5.5), and 100µL of H_2Od. The reaction was monitored by the increase of absorbance up to 405 nm due to the releasing of p-nitroanilide. Readings were taken every 15 seconds during 3 minutes. The p-nitroanilide extinction coefficient in these conditions was 8.33 $cm^{-1}\cdot(mmol/L)^{-1}$. During pH stability studies of soluble and immobilized enzymes the activity buffer was replaced with the same buffer used during the stability studies (50 mM sodium acetate buffer (CH_3COONa) pH 4-5, 50 mM Na_2HPO_4 pH 6-7, 50 mM Tris-HCl pH 8, 50 mM sodium bicarbonate buffer ($NaHCO_3$) pH 9-11).

Specific Enzymatic Activity (Spec EA): It was calculated as the ratio between the enzymatic activity and the protein concentration, and it was expressed in units of enzymatic activity (U) per mg of protein.

Ligand Interacting Groups Reactivity (LIGRe) is defined as the proportion between deprotonated (active) and protonated (inactive) ligand surface groups at immobilization pH (Torres-Salas *et al.*, 2011; Monte-Martinez and Cutiño-Avila, 2012). Theoretical bases for this calculation are considered on the classical Henderson-Hasselbalch equation (Equation 1) (Henderson, 1908; Hasselbalch, 1917).

$$pH = pKa + \log\left(\frac{A^-}{AH}\right) \quad (1)$$

Where pH is the immobilization pH, pKa is the acidic constant of the analyzed group, A^- and AH represents the deprotonated and protonated species, respectively. *LIGRe* is the relation (A^-/AH) (Equation 2), and the pKa values of protein's ionizable groups were calculated with the program PROPKA (Li *et al.*, 2005). *LIGRe* indicates the proportion among reactive and non reactive groups on the ligand surface at the immobilization pH.

$$LIGRe = 10^{(pH - pKa)} \quad (2)$$

Reactivity Index (RI) is defined as the probability of reaction of a single protein reactive group with the activated support (Equation 3).

$$RI = \frac{LIGRe}{LIGRe + 1} \quad (3)$$

Support Interacting Residues (SIR) are defined as the number of support interacting residues available to interact with at least the 10% of the ligand total area (so called Ligand Interacting Area, LIA) (Equation 4) (Cutiño-Avila *et al.*, 2013).

$$SIR = \frac{AG \times N_A \times 4\pi(MD/2)^2 \times 0.1}{S_{BET}} \quad (4)$$

Where AG is the activation grade of the support, N_A is the Avogadro´s number, MD is the protein diameter, and S_{BET} is the support surface area.

Theoretical Maximum Quantity (tMQ) is defined as the maximum protein quantity to immobilize per g of support (Torres-Salas *et al.*, 2011). In equation 5 MM is the protein molecular mass in Da and *mMQ* is the molar maximum protein quantity expressed in **µ**mol per g of support.

$$tMQ = mMQ \times MM \quad (5)$$

Estimated Maximum Quantity (eMQ) is defined as the maximum protein quantity that could be immobilized per g of support. It is a correction of *tMQ* conside-

ring the relationship between support pore diameter (PD) and maximum protein diameter (MD), in order to quantitatively estimate how diffusional restrictions could affect the maximum protein quantity to immobilize (Cutiño-Avila *et al.*, 2013). *eMQ* is calculated according to equation 6, where *OEC* is the Operational Effectiveness Coefficient.

$$eMQ = tMQ \times OEC \quad (6)$$

Practical Maximum Quantity (pMQ) is defined as the protein maximum quantity that has immobilized in the practice when a study charge is made (Equations 7 and 8). When increments on protein load do not represent an increase of the differential immobilisation grade (*diff.IG*) (del Monte *et al.*, 2002), but it remains stable within a 3% of variability, that *diff.IG* is considered the pMQ. *diff.IG* is determined taking into account the initial and final protein concentration of each immobilisation process, it is expressed in mg of protein immobilised per gram of support [mg/g] (equation 9).

$$diff.IG = \frac{mg\ Prot.total_{(Initials)}}{V_S} - \sum \frac{mg\ Prot.total_{(F+W)}}{V_S} \quad (7)$$

$$\frac{diff.IG_i - diff.IG_{i+1}}{diff.IG_i} < 0.03 \quad (8)$$

$$pMQ = diff.IG_i \quad (9)$$

Results and discussion

The covalent immobilization of bromelain and papain was carried in Glyoxyl-Sepharose CL 4B at different immobilization pH. To reach an optimum performance of the immobilized derivatives was applied the Rational Design of Immobilized Derivatives strategy using the program $RDID_{1.0}$. Were optimized and characterized two of the most relevant factors that influence over immobilization: immobilization pH and enzyme loading.

Predicting the most Probable Configuration

To determine the optimum immobilization pH and to predict the most probable configuration of the immobilized derivative was calculated the parameter *RI* (tables 1 and 2). For bromelain and papain in the three analyzed pH conditions, the highest probability of reaction with the activated support corresponds to the N-Term residue (N-Term). The configuration acquired by the immobilized derivative via the N-Term is catalytically competent in both cases, because the active site is orientated towards the pore´s lumen (figure 1-A1 and figure 1-B1).

When selecting the optimum immobilization pH, besides that the most probable configuration must be catalytically competent, we look for raising the pH, and consequently increase the reactivity of the protein interacting groups. The goal of these augment of reactivity is searching for multipoint covalent attachment and, in this way, considerably augment the stability of the immobilized biocatalyst.

Table 1. Reactivity Index (*RI*) of the interacting groups of immobilized bromelain in Glyoxyl-Sepharose CL 4B.

Residue	*RI* (pH 8.0)	*RI* (pH 9.0)	*RI* (pH 10.0)
N-Term	0.10	0.99	100
Lys40	0	0.06	0.41
Lys79	0	0.06	0.41
Lys179	0	0.05	0.37
Lys97	0	0.04	0.33
Lys90	0	0.04	0.30
Lys42	0	0.03	0.27
Lys144	0	0.03	0.27
Lys43	0	0.03	0.27
Lys59	0	0.03	0.24
Lys127	0	0.03	0.24
Lys64	0	0.03	0.24
Lys174	0	0.03	0.24
Lys175	0	0.03	0.24
Lys93	0	0.03	0.24

For both enzymes at pH 8.0 the *RI* of the N-Term is only the 10% (tables 1 and 2); while at pH 9.0 and 10.0 is almost 100%. At pH 9.0 increases the reactivity of several ε-amine of lysine (ε-Lys) but with an interaction probability to low (*RI* < 5%) (tables 1 and 2) and, consequently the most probable configuration remains via the N-Term. Nevertheless, at pH 10.0 are increased the *RI* of these ε-Lys (*RI* > 30%), as a consequence is enhanced the probability to obtain configurations with these residues within. Several of these ε-Lys are located in close proximity to the active site entry, which affects the catalytic efficiency of the immobilized derivative as shown in figure 1-A2 and figure 1-B2. Thus, RDID predictions lead to select pH 9.0 as the optimum immobilization pH.

The experimental results validate the predicted optimum immobilization pH. To corroborate this predictions the same amount of protein (2 mg/g Supp) was immobilized at three different pH (8.0, 9.0 and 10.0). Then the results were compared to determine which one of them showed the highest catalytic performance. In figure 2 are compared the catalytic efficiency of the three immobilized derivatives. The immobilized derivatives at pH 9.0 were more active than their homolog at pH 8.0 and 10.0. The difference is less noted between pH 8 and 9, because in both cases the most probable configuration acquired by the derivative is via the N-Term (tables 1 and 2). However, at pH 9.0 various ε-Lys are located around the N-Term, stabilizing

this configuration, and achieving a better orientation of the active center towards the pore´s lumen, resulting favorably in the ligand-substrate interaction, and as a consequence the enzymatic activity is increase.

Table 2. Reactivity Index (*RI*) of the interacting groups of immobilized papain in Glyoxyl-Sepharose CL 4B.

Residue	*RI* (pH 8.0)	*RI* (pH 9.0)	*RI* (pH 10.0)
N-Term	0.10	0.99	100
Lys10	0.01	0.10	0.53
Lys106	0.01	0.09	0.49
Lys39	0	0.05	0.37
Lys211	0	0.05	0.37
Lys100	0	0.05	0.33
Lys156	0	0.04	0.30
Lys190	0	0.03	0.24

Analysis of the possibilities of Ligand- Support Multipoint Covalent Attachment

In covalent immobilization the multipoint-covalent attachment enzyme-support increases considerably the stability of immobilized proteins. Multipoint-covalent attachment increases rigidity of immobilized enzymes inducing a higher resistance to conformational changes promote by heat, organic solvents, denaturizing agents, etc. (Guisán *et al.*, 1993; Mateo *et al.*, 2005; Mateo *et al.*, 2006). The probability of enzyme-support multipoint covalent attachment depends on the amount of carbonyl groups of the support available in the interaction zone with the ligand (*SIR*) and the number of interacting residues of the protein (*LIGRe*).

Was calculated the parameter *SIR* for each enzyme, considering a maximum activation grade of the support (71.42 μmol CHO/g Supp) (Guisán, 1988). The results for bromelain (*SIR* =21) and papain (*SIR*=14) indicate the existence of several carbonyl groups in the interaction area enzyme-support (10% of the ligand area). Theoretically can be formed 21 and 14 support-ligand bonds for t bromelain and papain, respectively. The results of *LIGRe* predictions were 15 reactive groups in (N-Term and 14 ε-Lys) for bromelain (table 1) and 8 groups (N-Term and 7 ε-Lys) for papain (table 2). This availableness of enzyme interacting groups in the surface conduces to the obtainment of multi-bounded immobilized derivatives. However, it is necessary to point out that these groups may not be localized inside the same interaction area (figure 3), defined in this case by the most probable configuration (figure 1-A1

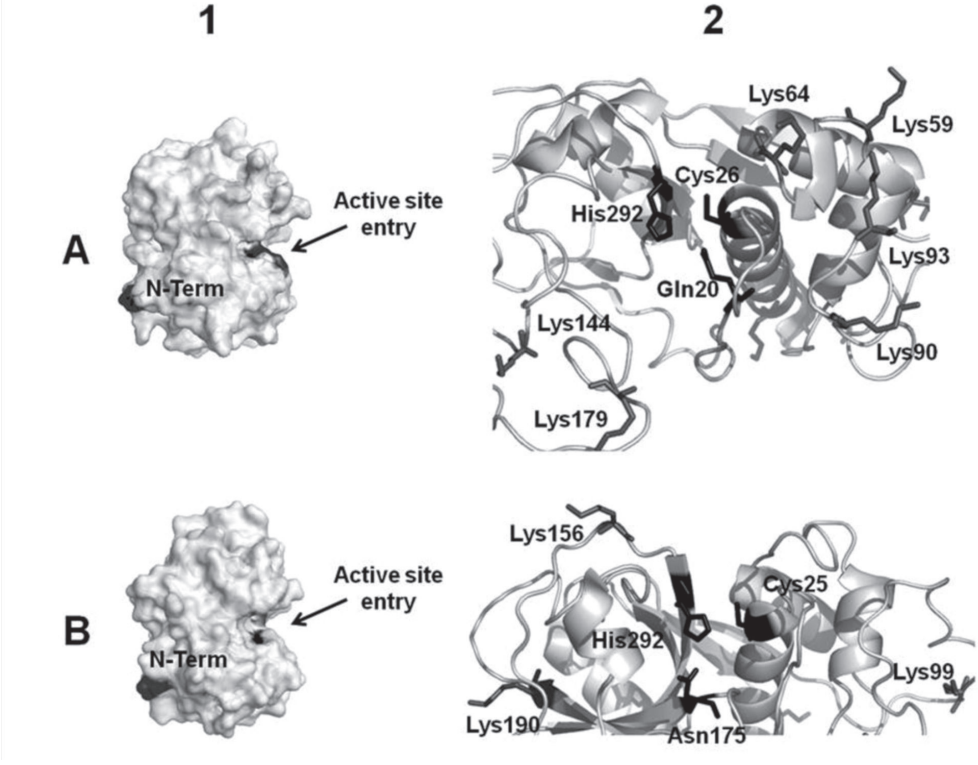

Figure 1. *RDID*$_{1.0}$ program predictions. A) Bromelain. B) Papain. 1) Most probable configuration of the immobilized derivative through the N-Term. 2) In black the active site residues according to Dunn (1989) and Lecaille *et al.* (2002) for bromelain and papain, respectively. In dark grey ε-Lys that can affect the catalytic activity of the immobilized derivative.

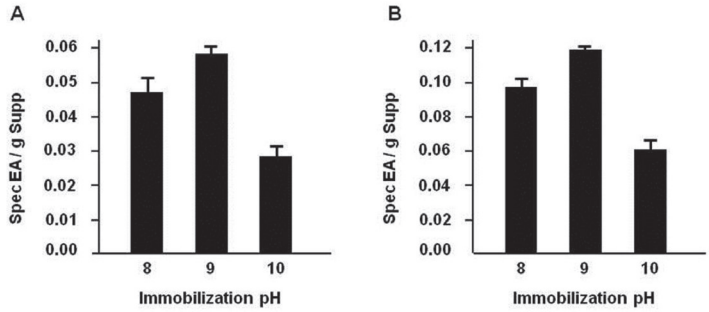

Figure 2. Specific enzymatic activities as function of immobilization pH. **A)** Bromelain immobilized derivatives at pH 8.0, 9.0, and 10.0. **B)** Papain immobilized derivatives at pH 8.0, 9.0, and 10.0.

and 1-B1), in both proteins via the N-Term. This result reduce from 15 to 5 (N-Term, Lys39, Lys42, Lys43, Lys127) for bromelain and from 8 to 5 (N-Term, Lys10, Lys39, Lys106, Lys211) for the papain the number of probable bonds with the support (figure 3).

For both enzymes the possibility of multipoint-covalent attachment with the support is viable (5 attachments), but there is differences in the residue´s *RI* involved in the union to the support for each enzyme. The presence, in the interacting area support-enzyme of papain, of two residues (ε-Lys10, ε-Lys106) with a *RI* ~ 0.10, increase the velocity of multipoint-covalent attachment and the efficiency on the stabilizing effect (figure 3).

Stability of soluble and immobilized enzymes

The effects of pH on stability of soluble and immobilized enzymes are shown in figure 4. Soluble bromelain was highly stable at pH range 6-9 retaining 80% residual activity after 2 h incubation, while at acid and basic pH stability was severely affected (figure 4A). In contrast, immobilized bromelain showed great stability at pH 4-11, keeping up to 60% residual activity (figure 4B). But the highest stability was raised at pH 7.0, 95% residual activity after 2 h incubation (figure 4B).

The stability of papain was dramatically enhanced after immobilization in Glyoxyl-Sepharose CL 4B support. Soluble papain showed residual activity up to 80% only at pH 7-8, stability decrease considerably at acid and alkaline pH (figure 4C). These results are consistent with the report of Liu and Hanzlik (1993), which demonstrate that papain showed highest stability at physiological pH values. Immobilized papain was extremely stable, retaining up to 50% residual activity after 2 h at pH 4-11 (figure 4D). Better residual activities were achieved at pH 7-9 (up to 90% residual activity in all cases). However, the highest stability was raised at pH 8.0, 100% residual activity after 2 h incubation.

The effects of temperature on stability of soluble and immobilized enzymes are shown in figure 5. The thermal stability of soluble bromelain was very low, with the residual activity being reduced to 70% from the initial activity after preservation for 2 h at 30 °C. A drastic loss in activity was observed in further increase of temperature (figure 5A). In contrast, immobilized bromelain showed 90% residual activity during a 2 h exposure to 30 °C, and retained up to 60% residual activity after incubation 2 h at 60 °C (figure 5B). On the other hand, soluble papain was very stable at 40 °C, with 87% residual activity.

The thermal stability of soluble papain was very high, retaining 80% and 60% residual activity after preservation for 2 h at 30 °C and 40 °C, respectively. Further increase of temperature caused stability loss to 20% residual activity. On the contrary, immobilized papain showed high thermal stability retaining 90% residual activity after 2 h incubation at 30 °C, and 60% residual activity at 60 °C.

Both soluble and immobilized CPA, incubated at 30-50 °C, showed to be more stable at pH 7.0-8.0. Figure 5 shows that the stabilization of the immobilized enzyme (ratio between the half-life of the immobilized enzyme and half-life of the soluble enzyme) was higher at neutral-alkaline pH values than at acidic pH values. The achieved stabilization levels suggest that multipoint covalent attachments have promoted a real rigidification of the overall enzyme structure. This rigidification process is global but may not affect in the same way all portions of the biomolecule. In addition, the inactivation process may follow different mechanisms for acidic or for neutral/alkaline pHs. These facts could explain the lower stabilization of the immobilized papain at acidic pH values.

Optimization of Protein Load

The selection of the support and the technique for the preparation of an optimal immobilized protein is dictated by the low diffusional resistance of the support in junction of with its ability to incorporate the optimal amount of protein per surface area.

To determine the optimum initial protein quantity in a load study, were calculated the parameters *tMQ* and *eMQ* using $RDID_{1.0}$ program. To demonstrate the accuracy of these predictions was carried out a load study,

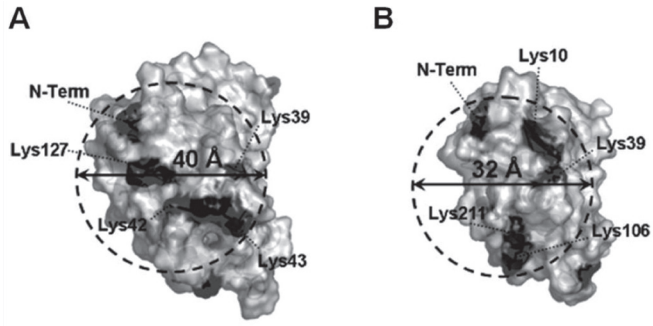

Figure 3. Ligand interaction areas with the support (LIA) in the most probable configuration. **A)** Bromelain. **B)** Papain. **(---)** LIA. **(↔)** LIA diameter in Å calculated with the program *RDID*$_{1.0}$. **(■)** Ligand interacting residues in LIA that define the most probable configuration.

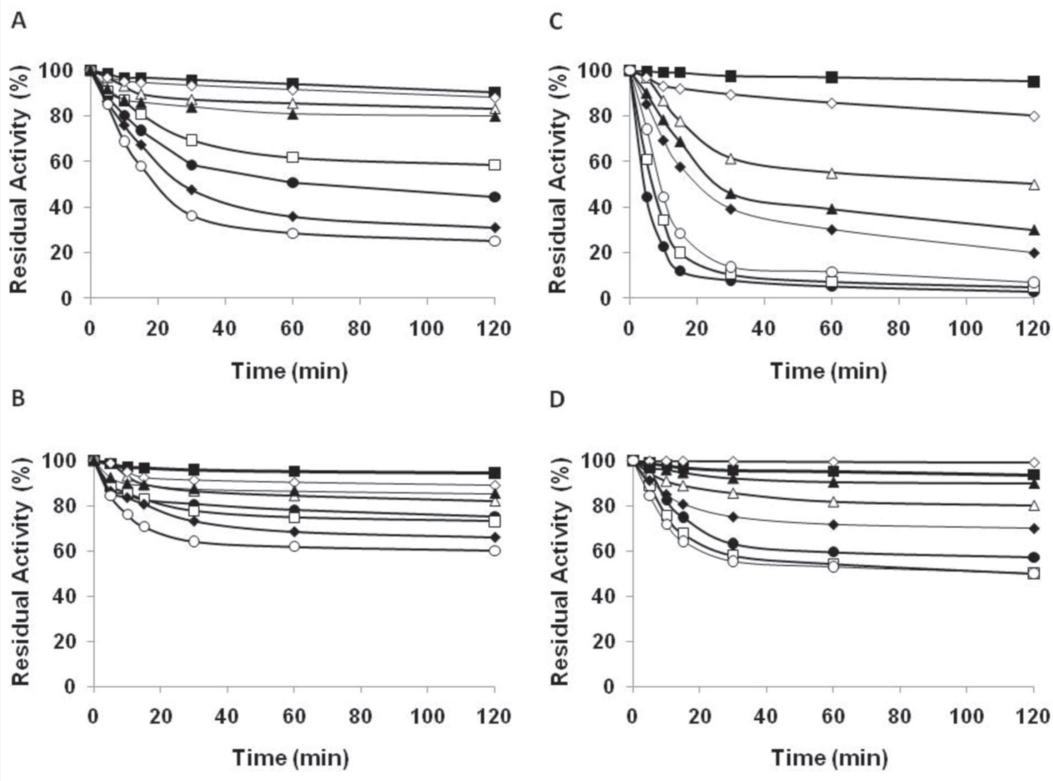

Figure 4. Effect of pH on stability of soluble and immobilized bromelain and papain (100% of the activity was the initial activity). **A)** Soluble bromelain after 2 h incubation. **B)** Immobilized bromelain after 2 h incubation. **C)** Soluble papain after 2 h incubation. **D)** Immobilized papain after 2 h incubation. **(●)** pH 4.0. **(□)** pH 5.0. **(Δ)** pH 6.0. **(■)** pH 7.0. **(◊)** pH 8.0. **(▲)** pH 9.0. **(♦)** pH 10.0. **(○)** pH 11.0.

was took as a reference the *eMQ* calculated, and 8 additional loads: 4 above and 4 below *eMQ*. The immobilization experiments were made at pH 9.0, which was the optimum immobilization pH validated, and they were followed by means of the determination of *diff.IG* for each assay. With the *diff.IG* values immobilization isotherms were constructed for each enzyme (figure 6). The isotherms allow evaluating how *diff.IG* behaves when protein charge increases.

As it can be seen in table 3, the *pMQ* values obtained experimentally were inferior to the predicted *tMQ*, but very similar to the *eMQ*. This is owing to *tMQ* are calculated assuming ideal conditions and restrictions to diffusion are not considered, this theoretical maximum value is only reached by very small molecules, as a result, *tMQ* is the maximum limit of protein to immobilize in a specific support. In the other hand, when calculating *eMQ* diffusional restrictions are taken into account over the protein quantity to immobilize. The immobilization isotherms corroborate these results. Both isotherms saturate at values nearly the predicted *eMQ*, and the inflection point indicates the maximum protein quantity that was immobilized in the practice. These results

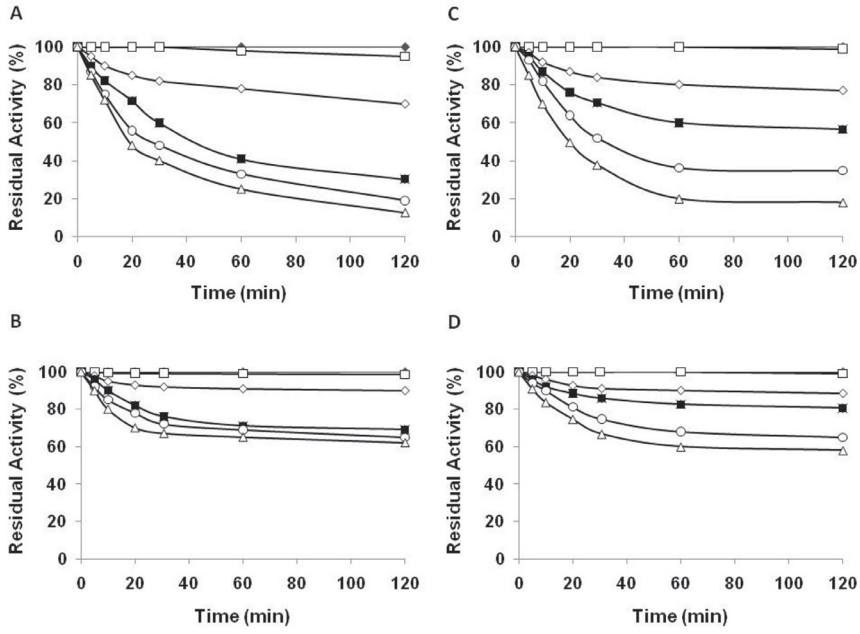

Figure 5. Effect of temperature on stability of soluble and immobilized bromelain and papain (100% of the activity was the initial activity). **A)** Soluble bromelain after 2 h incubation. **B)** Immobilized bromelain after 2 h incubation. **C)** Soluble papain after 2 h incubation. **D)** Immobilized papain after 2 h incubation. **(♦)** 10 °C. **(□)** 20 °C. **(◊)** 30 °C. **(■)** 40 °C. **(○)** 50 °C. **(Δ)** 60 °C.

demonstrate the precision of *RDID*$_{1.0}$ program´s predictions for optimization of protein load studies.

A critical requirement for any material to be used as a support for protein immobilization is the necessity to have a large surface area (small particle size or highly porous materials). The protein binding support capacity will depend on the pore diameter (PD) and tortuosity of the support, and also the protein size. This last one explains the highest adsorption capacity of papain showed by Glyoxyl-Sepharose CL 4B. Papain is 12Å smaller than bromelain and consequently; the binding capacity of the employed support is higher for papain (0.99µmol/g Supp and 0.6µmol/g Supp for papain and bromelain, respectively).

Conclusions

Optimization of immobilization conditions becomes as an important step to generate more robust and

Table 3. *RDID*$_{1.0}$ program´s predictions and experimental results for bromelain and papain immobilization in Glyoxyl-Sepharose CL 4B.

Enzyme	*tMQ* (mg/g Supp)	*eMQ* (mg/g Supp)	*pMQ* (mg/g Supp)
Bromelain	45.96	12.4	9.97 ± 0.29
Papain	47.66	15.7	15.51± 0.34

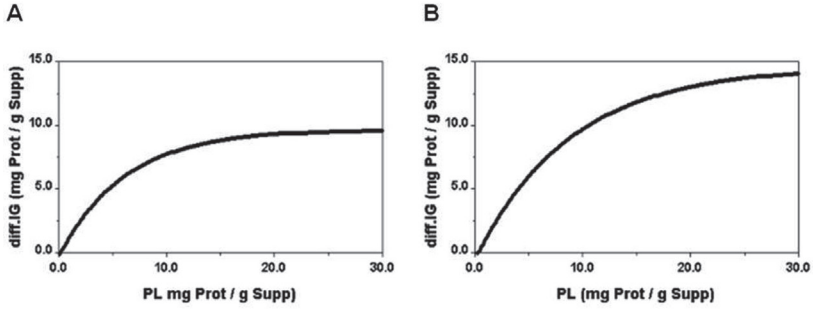

Figure 6. Immobilization Isotherms in Glyoxyl-Sepharose CL 4B at pH 9.0. **A)** Bromelain. **B)** Papain. **(PL)** Protein load to immobilize. **(*diff.IG*)** protein quantity adsorbed on the support.

efficient biocatalyst. The employment of enzymes in affinity chromatography and bioconversion processes often encounters the problem of inactivation of enzyme. Bromelain and papain immobilized derivatives showed desired characteristics for industrial biocatalysis, such as: elevate pH stability retaining 95% and 100% residual activity at pH 7.0 and 8.0, for bromelain and papain, respectively; high thermal stability at 30 °C retaining 90% residual activity for both immobilized enzymes; a catalytic configuration bonded by immobilization at optimal pH; and the ligand load achieve ensure the minimization of diffusional restrictions.

Glyoxyl-Sepharose CL 4B supports seems to be very suitable matrix to obtain stabilized enzymes via multipoint-covalent attachment, but not every immobilization protocol allows achieving this goal. Following is propose and optimized protocol for immobilization-stabilization of bromelain and papain in Glyoxyl-Sepharose CL 4B:

- Immobilization at pH 9.0 in 100mM of $NaHCO_3$ during 2hrs.
- *eMQ* value as initial protein load: 12.4 and 15.7 mg of protein for bromelain and papain, respectively.
- Maximum support activation grade: 71.4 µmol CHO/g Supp.

The employment of *RDID*$_{1.0,}$ program as the carrying out of the Rational Design of Immobilized Derivatives strategy allows selecting the best way to synthesize highly stabilized affinity matrix of bromelain and papain for protease inhibitors purification. *RDID*$_{1.0}$ predictions were highly accurate when comparing with experimental results in both cases. Besides, the use of this program permits improving the performance of the immobilized derivative in terms of catalytic efficiency.

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