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Evidence of structural changes of an enzymatic extract entrapped into alginate beads

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ABSTRACT

In this work, we analyzed the structural changes of *araujiain* entrapped into alginate beads. *Araujiain* is an enzymatic preparation containing three known enzymatic fractions with each fraction individually presenting a similar catalytic performance. Fluorescence and infrared spectroscopy, thermal analysis and residual catalytic activity studies were carried out. A small red shift in the spectrum of *araujiain* was observed after the entrapment process. Changes in the polarity around the tryptophan (Trp) residues were associated with an enzyme conformational change. From the Fourier transform infrared spectroscopy (FTIR) analysis, it was demonstrated that interactions between the enzyme extract and Ca alginate caused different structural behavior in *araujiain*. According to the diffuse reflectance infrared Fourier transform spectroscopy (DRIFT) study, it was possible to conclude that a secondary structure with a high α-helical character was responsible for the highest activity of entrapped *araujiain*. Finally, from thermal analysis measurements, it was proved that entrapment of *araujiain* augments the thermal stability of both the enzyme extract and Ca alginate, indicating a possible interaction between enzyme extract and its support.

1. Introduction

The successful application of biological entities (enzymes, antibodies and other proteins) in biotechnology depends on the ability to stabilize a biological component in an unnatural environment while retaining its function and activity. This stabilization is frequently achieved by entrapment or immobilization in organic and inorganic structures and has proved to be beneficial for protein digestion and separation, biosensors, biocatalysis, drug delivery and tissue engineering [1–9].

In particular, enzymes are versatile biocatalyst, possessing some excellent properties (such as high activity and chemio-, region- and stereospecificity) that render them able to perform various reactions under mild conditions. Among these, the use of partially purified enzymes as catalysts in multistep synthetic organic chemistry has become widespread [10–13]. Unfortunately, these properties are not always adequate for industrial applications [14]. Due to different working conditions used in reaction media and enzyme extraction steps, a loss of catalytic activity can be experienced in such applications. However, these disadvantages can be overcome by protein immobilization into a solid structure, increasing the protein stability [15,4]. This approach provides scope to enhance the ability of enzymes to catalyze specific reactions in a hostile environment.

The immobilization of enzymes using the entrapment process into biopolymers matrices can lead to increased enzyme stability, one of the key properties for industrial applications. The enzymes are retained within a confined space, with restricted conformational mobility that contributes to the observed stability enhancement [16,17]. In fact, it has also been shown that the entrapment process can protect enzymes against exposure to extreme pH conditions, and can prevent enzyme leaking during catalysis. These factors are important from both an economic and environmental standpoint, and have long been highlighted as important advantages of the entrapment process [18,19]. Among several supports employed for enzyme entrapment, alginate has shown suitable characteristics for this purpose. It also has interesting biological and chemical properties: it is biodegradable, biofuel-compatible, bioactive, nontoxic and may be chemically modified [20–22].

In a previous work, the entrapment of *araujiain* (partially purified cystein phytoprotease) within calcium alginate beads was carried out successfully and its application to biocatalysis was proved [23]. Partially purified enzymes or crude extracts are

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currently used, taking into account that, for industrial applications, enzyme purity is usually of secondary importance to the cost [14,24]. The entrapment process was demonstrated to improve the catalytic activity and operational stability of *araujiain*. In particular, the entrapped enzyme exhibited a higher activity and both thermal and storage stability. These results demonstrate that hydrogel carrier (alginate) is able to provide a protective microenvironment for the enzyme. Additionally, changes in *araujiain* catalytic activity could be addressed in the context of the enzyme conformational changes after entrapment.

This work deals with the study of structural changes using a partially purified enzyme extract (*araujiain*). We highlight changes of the enzyme microenvironment when the extract is entrapped in an alginate matrix. Both free and entrapped enzyme, were analyzed by fluorescence and FTIR spectroscopy and thermogravimetric analysis (TGA). Finally, the enzyme residual activity as a function of temperature was evaluated.

2. Material and methods

2.1. Materials

Araujiain is a partially purified enzymatic preparation obtained from the latex of fruits of *Araujia hortorum* Fourn. (*Asclepiadaceae*). This preparation contains three cysteine proteases (*araujiain* hI, hII and hIII) belonging to the papain family. Such proteases have previously been biochemically studied in detail [25,26]. The Laboratorio de Investigación de Proteínas Vegetales (LIPROVE) kindly provided the enzyme extract which was prepared according to Priolo et al. [25].

All chemical used in this work were analytical grade and supplied by Sigma (St. Louis, USA).

2.2. Proteolytic activity assays

The measurement of the proteolytic activity (free and immobilized *araujiain*) was carried out using N-α-benzoyl-DL-arginine 4-nitroanilide (BAPNA) as substrate (5 mg/mL). After 120 min of incubation in buffer Tris–HCl (0.1 M, pH 8.5) at different temperatures (37–70 °C), the absorbance of the *p*-nitroaniline released was measured spectrophotometrically at 410 nm. Enzymatic units (IU) were obtained by performing a standard curve of *p*-nitroaniline. The residual activity was measured in term of the relative activity, which was calculated as the percentage ratio of specific activity at a given temperature (after 120 min of incubation) to the specific activity at the optimum temperature (after 5 min of incubation). Variation coefficients $((\text{Sd Mean}^{-1}) \times 100)$ of reported values were less than 1.5% for activity assays, calculated in each case from triplicate results.

2.3. Immobilization of araujiain within alginate beads

Araujiain was immobilized within calcium alginate beads (using sodium alginate of viscosity 20,000–40,000 cps). Sodium alginate solution was prepared in distilled water and the final concentration was adjusted at 2% (w/w) considering that using this concentration, the maximum yield of immobilization was obtained [23]. Beads were formed by dropping sodium alginate solution (15 mL) containing *araujiain* (15 mg/mL sodium alginate solution) through a syringe fitted with a needle of 1 mm diameter into $CaCl_2$ solution (200 mL, 0.1 M). The solution was gently stirred for 30 min to avoid beads deformations. Then, beads were collected, washed with distilled water and stored at 4 °C. The amount of protein was estimated by known Bradford dye binding method using serum albumin as the standard protein. The protein content of the immobilized *araujiain* was calculated by subtracting the amount of free protein at the end of the immobilization process from the protein originally added. Whereas, the yields of immobilization was calculated as the ratio of the activity of immobilized enzyme to the activity of the free enzyme used. High protein content of the immobilized *araujiain* (~98%) and high yields of immobilization (~91%) were obtained.

2.4. Fluorescence measurements

Tryptophan (Trp) fluorescence spectra were measured at 25 °C using a Shimadzu RF-5301PC spectrofluorimeter (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan) equipped with a Xenon discharge lamp and solid sample holder. The excitation and emission spectral bandwidths used were 1.5 nm. Samples were excited at 295 nm to minimize the emission arising from tyrosine residues, while the emission spectrum was recorded between 300 and 450 nm. Results were reported as the means of studies performed in triplicate.

The free *araujiain* was analyzed in aqueous solution (200 μl/mL), while the entrapped *araujiain* was analyzed as film. The film was prepared containing *araujiain* in relation to 15 mg/mL alginate (2%) in the precursor solution. 10 mL of *araujiain*/alginate solution was poured into a Petri dish. 30 mL $CaCl_2$ 0.1 M solution was further added. After consolidation (2 h), the obtained film was washed with distilled water and the film was then stored in an oven at 50 °C. The film thus obtained has a thickness of 50 μm and was measured with a Köfer Micrometer (accuracy ± 1 μm).

2.5. Infrared spectroscopy

The diffuse reflectance infrared Fourier transform spectroscopy (DRIFT) and Fourier transform infrared spectroscopy (FTIR) spectra were measured at 25 °C using Nicolet Protégé model 460 spectrophotometer, provided with CsI beam splitter between 4000 and 225 cm^{-1}. A total of 128 interferograms, at a resolution of 4 cm^{-1}, were acquired. DRIFT samples were analyzed as powder (free enzyme extract was obtained as powder, while the blank beads and enzyme entrapped were ground to fine powder after consolidation, and were stored in dry conditions at 4 °C). DRIFT analyses were carried out based on at least five replicate measurements, obtaining 95% of reproducibility.

For measuring FTIR, a total of 3% (w/w) of the sample (blank beads, free and entrapped enzyme), with respect to the potassium bromide (KBr) disc, was mixed with dry KBr and annealed into a disc using a hydraulic press. This process has previously been shown not to alter IR spectra of proteins [27,28]. The basis line was corrected using the OMNIC spectrophotometer program and the characteristic peaks were recorded.

2.6. Thermal analysis

Dynamic weight loss tests were conducted on a thermogravimetric analyzer Shimadzu TA51 (TGA) and a differential thermal analyzer Shimadzu TA50 (DTA). Samples were ground to fine powder and measurements were performed using a temperature range of 25–400 °C at a heating rate of 10 °C/min, using N_2 as purge (20 mL/min) and air as reaction gas (50 mL/min). Alumina was used as a reference standard to the DTA analysis. For both DTA and TGA, Pt pan was used.

3. Results and discussion

3.1. Fluorescence measurements

It has been reported that the maximum emission wavelength of a fluorophore depends on the environmental polarity around the molecule [29]. Therefore, the fluorescence signal can be used as an

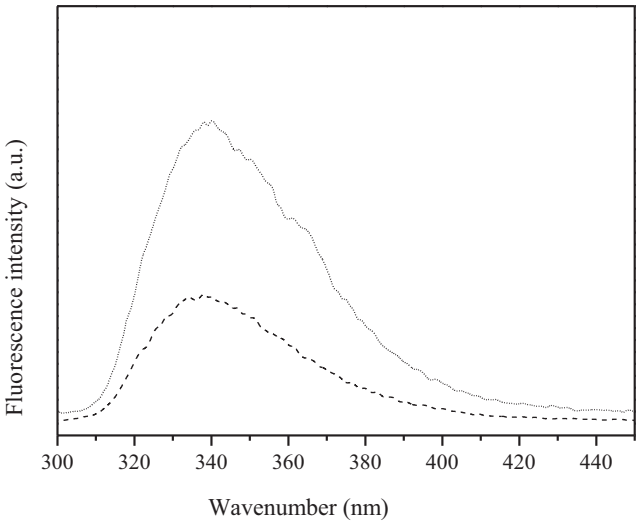

Fig. 1. Fluorescence emission spectra of free (dash dot line) and entrapped *araujiain* (solid line). Both samples were excited at 295 nm and the spectra were obtained at 25 °C.

optical probe to analyze the effect of the microenvironment on the protein tertiary structure containing Trp [30]. Although the structural basis of *araujiain* has not yet been elucidated, it is known that papain-like cystein proteases, including *araujiain*, usually have at least one Trp residue in their N-terminal sequence [25,26]. The Trp fluorescence spectra of free and entrapped *araujiain* are shown in Fig. 1. *Araujiain* showed a fluorescence emission band when was excited with a radiation at 295 nm, proving that at least one Trp residue is present in the protein molecule. As can be observed, the emission maximum of the free enzyme was at 335 nm. After the entrapped process, there was a small red shift of the fluorescence and an emission maximum at 340 nm was observed. It is important to note that the entrapped enzyme has a higher local concentration that the free enzyme solution, causing a higher intensity band.

Changes in emission spectra from Trp can be seen in response to protein conformational transitions, subunit association, ligand binding or denaturation, all of which can affect the local environment surrounding the indol ring [29]. The small red shift in the spectrum of the entrapped form of the enzyme indicates that Trp residues have more polarizable surroundings [31]. Changes in the polarity around the Trp residues may be associated with a conformational change of the enzyme. It is a reasonable assumption that a structural modification of an enzyme alters its catalytic activity.

3.2. Infrared spectroscopy

The alginate gel confines the enzyme to a restricted space. The interaction of the enzyme with the functional groups on the surface of beads, or large areas of contact between enzyme and support, have the potential to alter protein function in several ways, including changes in protein conformation (secondary and tertiary structures) [32]. FTIR spectroscopy was carried out in order to detect interactions between *araujiain* and alginate.

Fig. 2 gives FTIR spectra of calcium alginate beads (blank beads), free *araujiain* and *araujiain* immobilized within calcium alginate beads within the wavenumbers ranges of 4000–500 cm^{-1}. Characteristic bands appeared in the alginate beads spectrum at 3447 cm^{-1} due to hydroxyl groups, and at 1639 and 1434 cm^{-1} due to asymmetric —COO^- stretching vibration and symmetric —COO^- stretching vibration, respectively (Fig. 2a), while peaks

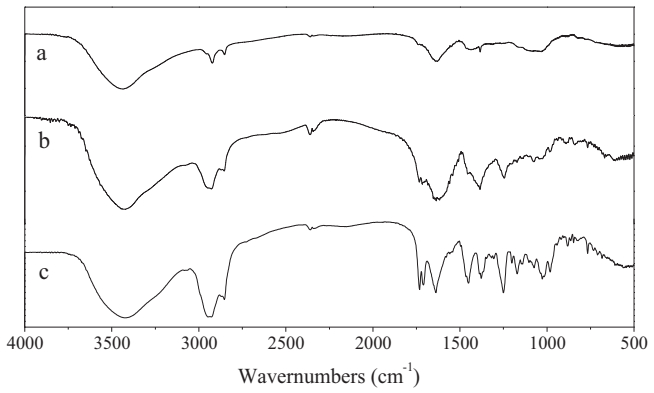

Fig. 2. FTIR spectra of a: calcium alginate beads (blank beads), b: free *araujiain* and c: entrapped *araujiain*.

around 1036 cm^{-1} are attributed to the stretching of C—O—C. *Araujiain* spectrum showed a broader band at 3422 cm^{-1} due to the O—H and N—H stretching vibration, and also a band at 2926 cm^{-1} with a shoulder at 2854 cm^{-1}, both due to C—H stretching vibration (Fig. 2b). As is already known, the spectrum region that originates from the carbonyl stretching of the peptide group shows bands associated with the secondary structure of a protein [27]. Accordingly, two defined bands at 1731 and 1716 cm^{-1} and a broad band that included narrow peaks at 1649, 1639 and 1619 cm^{-1}, representing the secondary structure of proteins, were observed in the free *araujiain* spectrum (Fig. 2b).

The entrapped *araujiain* showed a similar spectrum to free form in the wavenumber ranges of 4000–2000 cm^{-1}, but with broader bands and shifted towards higher wavenumber than free enzyme extract (Fig. 2c). In contrast, bands at 1731 and 1716 cm^{-1} in the free form spectrum were shifted to a lower wavenumber (1725 and 1705 cm^{-1}, respectively) and these showed higher intensity after entrapment (Fig. 2c). These shifts and intensity band changes are probably due to the hydrogen bond. The covalent bonds in the donor and acceptor groups are weaker, while the energy barrier for angle deformation becomes higher. Hence, in the groups that are involved in the hydrogen bond formation, vibration frequency decrease with the simultaneous increase in the vibrations deformation frequency [33].

The above analysis demonstrated that interactions between the enzyme and alginate after the immobilization process caused different structural behavior of *araujiain* and, consequently, its catalytic activity was increased.

Structural information is obtained by analysis of the conformationally sensitive amide I band, which is located between 1600 and 1700 cm^{-1} [27,34–37]. This band is present due to the in-plane C=O stretching vibration, weakly coupled with C–N stretching and in plane N–H bending [37]. Each type of secondary structure (i.e. α-helix, β-sheet, β-turn and disordered) gives rise to different C=O stretching frequencies, resulting in characteristic band positions, which are designated by wavenumber (cm^{-1}).

In order to improve the analysis of the secondary structure of *araujiain*, avoiding the use of deconvolution of the band at 1700–1600 cm^{-1}, DRIFT spectroscopy was used. Thus, throughout the amide band I of the DRIFT image for both free and entrapped *araujiain*, we were able to predict the distribution of the secondary structure. In Fig. 3, the spectra of both enzyme forms can be observed. The maximum intensity of the free *araujiain* spectrum was found at 1633 cm^{-1} (β-sheet structure), followed in intensity by a band at 1653 cm^{-1} (random structure) and a band at 1683 cm^{-1} (β-turn structure). The entrapped *araujiain* spectrum showed a band of maximum intensity at 1653 cm^{-1} (random structure) followed by two bands of lower intensity at 1647 and 1683 cm^{-1}

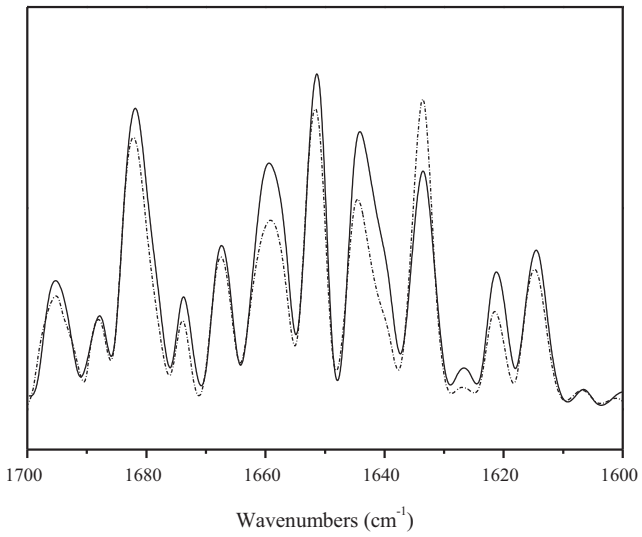

Fig. 3. DRIFT spectra of free (dash dot line) and entrapped *araujiain* (solid line).

(random and β-turn structure, respectively). The frequency assignment for the amide I band components were performed according to methodology described in a previous study [27].

Araujiain is an enzymatic preparation containing three fractions (hI, hII and hIII). Each fraction individually presents a similar catalytic performance to the enzymatic preparation [25,26]. Furthermore, the infrared spectral signals are an average contribution of the all protein molecules in the sample. Therefore, the above analysis can be used to provide information about conformational changes in the *araujiain* after the entrapment process.

After the immobilization process, an over 1.5-fold increase in the percentage of the α-helix component and a decrease in the percentage by over 1.7-fold of the β-sheet component were observed. Larger amounts of antiparallel β-sheet residues indicated the formation of tight intermolecular hydrogen bonds and larger quantities of enzymatic aggregates in the free enzyme. These results agreed with fluorescence studies, indicating an enzymatic conformational change in alginate matrix. The results from IR allowed us to conclude that the protein secondary structure has been modified, evidencing an increase of catalytic activity after entrapment process [23].

The analysis of second derivative allows for the analysis of similarity between two spectra. Prestrelski et al. have developed a mathematical procedure to calculate the spectral correlation coefficient (similarity, r) between two second derivative spectra [37]. Such correlation coefficient between two spectra of a given protein is 1 when there are no conformational changes in the protein. When conformational changes occur, the spectral differences are significant and the coefficient r becomes lower. A coefficient $r = 0.77$ was obtained for the relation between the free and entrapped *araujiain* spectrum.

Although the correlation between enzyme structure and catalytic activity requires direct measurement of both active-site structure and the effect of the reaction medium on the transition state of the reaction, it is possible that the non-covalent forces which maintain the native secondary structure of *araujiain* could have been modified when it was immobilized [38–41].

3.3. Thermal analysis

Fig. 4 shows the typical weight loss (TGA) and derivative of weight loss (DTA) curves of the three samples: blank beads, free *araujiain* and entrapped form. Analyzing Fig. 4a, a first mass loss in the blank beads can be observed, which can be ascribed to the elimination of free water. Then, an important endothermic process (onset 181 °C) with a mass loss of 24.92% was observed. In a recent work, Ross et al. have reported the thermal degradation of alginate acid, Ca and Na alginate [42]. They found that the temperatures at which devolatilization starts are 160, 200 and 220 °C for alginate acid, Ca and Na alginate, respectively. The onset at 181 °C in Fig. 4a indicates that blank beads can be a blend of alginic acid and Ca alginate. At 285 °C an exothermic process with mass loss of

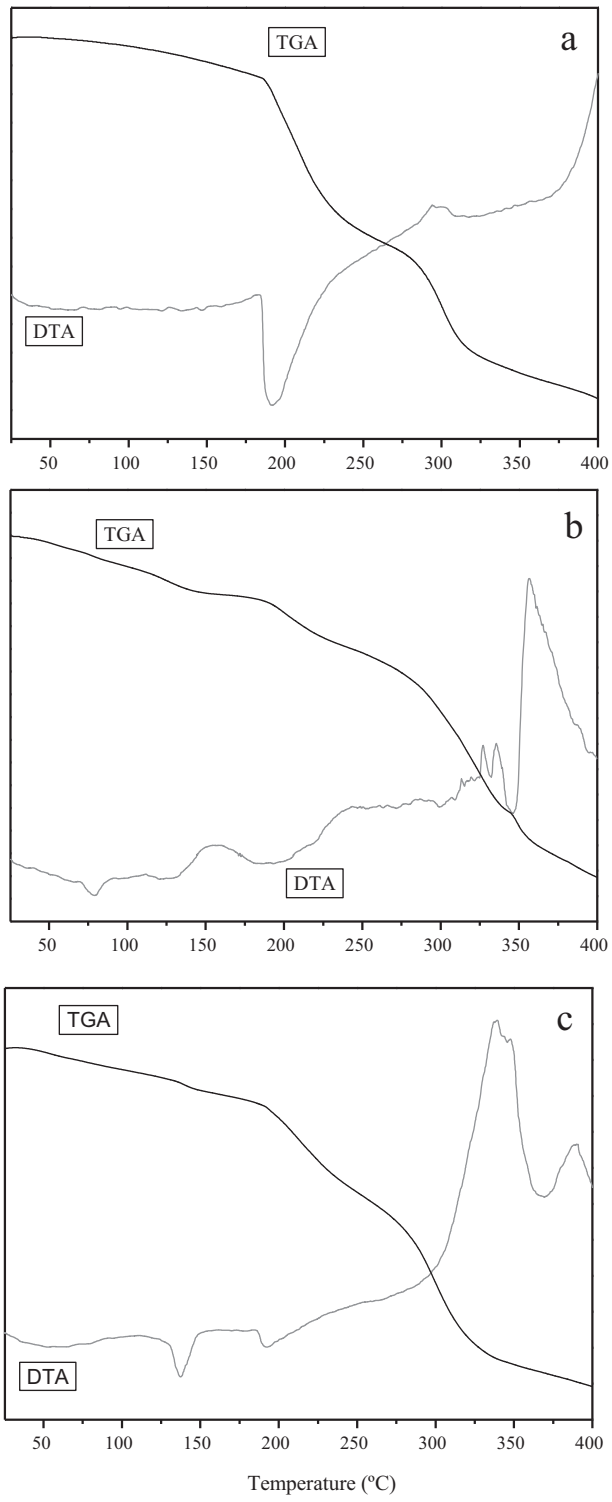

Fig. 4. Thermogravimetric curves (TGA and DTA studies) of a: blank beads, b: free *araujiain* and c: entrapped *araujiain*.

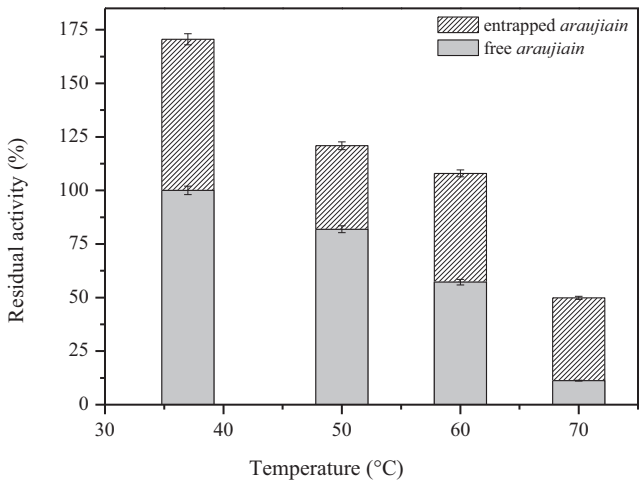

Fig. 5. Residual activity of both free and entrapped *araujiain*, after 120 min of incubation at different temperatures (37–70 °C).

19.37% can be observed. Lu et al. studied the thermal decomposition of calcium carboxylic acid salts [43]. These authors report that beyond 275 °C all compounds analyzed show an exothermic peak corresponding to $CaCO_3$ formation. This could also correlate to the thermal event shown in Fig. 4a at 285 °C. The course of $CaCO_3$ formation was reported to occur between 275 and 525 °C [43]. It can at least be observed that Ca alginate also continues its decomposition through $CaCO_3$ formation with a new exothermic event (onset 390 °C).

The mass loss of free *araujiain* began at 70 °C, probably due to loss of a volatile compound. Then two endothermic events were present at 109 and 208 °C, respectively (Fig. 4b). Also, exothermic events occurred beyond 322 °C until total decomposition. Instead, the mass loss of the entrapped enzyme started at 128 °C (Fig. 4c). The next endothermic process (onset 185 °C) can be attributed to the beginning of decomposition (devolatilization) of calcium alginate. When enzyme is entrapped in Ca alginate, the onset of $CaCO_3$ formation temperature reached 306.5 °C (against 285 °C of blank beads) and an exothermic protein decomposition event began at 374 °C (in free enzyme extract this event occurred at 322 °C). These results showed that the entrapment of *araujiain* augments the thermal stability of both the enzyme extract and Ca alginate, indicating a strong interaction between *araujiain* and alginate matrix.

It is known that the process of immobilization into a solid support can influence the thermal stability of an enzyme. As shown in Fig. 5, the entrapped *araujiain* showed a higher catalytic activity after 120 min of incubation at different temperatures (37, 50, 60 and 70 °C). The matrix of alginate was able to protect *araujiain* and showed ~50% of initial residual activity after 120 min of incubation at 70 °C. This value increased over 4.44-fold when compared with the free form. Moreover, it was noticeable that thermal event of 70 °C measured in free enzyme extract (Fig. 4b) was not due to protein denaturalization (Fig. 5). It is important to point that the residual activities were calculated from specific activities for each enzymatic form and the residual activity difference is not because of the difference in the amount of enzymes (both entrapped and free form) used in the activity assays.

Most proteins lose their biological activity to some degree during a physical or chemical modification step such as encapsulation. However, each protein, due to its unique tertiary and quaternary structure, responds differently to the modification steps and alterations in its environment [38]. In this sense, *araujian* appears to be a robust protein with respect to the entrapment process. Similar results have been reported in the literature for others immobilized enzymes [44,45].

4. Conclusion

In this work, structural changes using a partially purified enzyme extract (*araujiain*) entrapped into alginate beads were analyzed using fluorescence and infrared spectroscopy studies. After the entrapment process, a small red shift in the spectrum of the entrapped enzyme form was observed. Changes in the polarity around the tryptophan residues were associated with a conformational change of the enzyme extract. According to a study of the secondary structure using DRIFT spectroscopy, it was possible to conclude that a secondary structure with a high α-helical character had formed. In addition, from the spectroscopy analysis, it was demonstrated that interactions between the enzyme and alginate were the cause of the different structural behavior of *araujiain*. Consequently, its catalytic activity was increased. This is in marked contrast to other carrier systems that usually lead to a decrease of enzymatic activity.

From thermal analysis measurements it was proved that the entrapment of *araujiain* in Ca alginate augments the thermal stability of both enzyme extract and Ca alginate. These results indicated a possible interaction between the enzyme extract and alginate matrix. Evidence of a favorable structural change for the enzyme extract when entrapped in Calcium alginate was demonstrated by this study.

Finally, it was demonstrated that hydrogel carrier (alginate) is able to provide a protective microenvironment for *araujiain* by mean of a structural change improving their catalytic activity and operational stability. In addition, *araujian* showed robustness with respect to the entrapment process. Accordingly, this catalyzer can be used for both hydrolytic and synthetic applications.

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References

[1] L. Qiao, Y. Liu, S. Hudson, P. Yang, E. Magner, B. Liu, A mesoporous reactor for high throughput proteolysis, Chem. Eur. J. 14 (2008) 151–157.
[2] Y.-J. Han, G.D. Stucky, A. Butler, Mesoporous silicate sequestration and release of proteins, J. Am. Chem. Soc. 121 (1999) 9897–9898.
[3] X. Hu, S. Spada, S. White, S. Hudson, E. Magner, J.G. Wall, Adsorption and activity of a Domoic acid binding antibody fragment on mesoporous silicates, J. Phys. Chem. B 110 (2006) 18703–18709.
[4] R.A. Sheldon, Enzyme immobilization: the quest for optimum performance, Adv. Synth. Catal. 349 (2007) 1289–1307.
[5] V.I.I. Slowing, B. Trewyn, S.-Y. Lin, Mesoporous silica nanoparticles for intracellular delivery of membrane-impermeable proteins, J. Am. Chem. Soc. 129 (2007) 8845–8849.
[6] K.Y. Lee, S.H. Yuk, Polymeric protein delivery systems, Prog. Polym. Sci. 32 (2007) 669–697.
[7] X. Wang, L.X.S. Chen, Z. Zhu, J. Zhao, J.-M. Chovelon, N.J. Renaul, Tyrosinase biosensor based on interdigitated electrodes for herbicides determination, Int. J. Electrochem. Sci. 1 (2006) 55–61.
[8] H. Fan, Y. Hu, C. Zhang, X. Li, R. Lv, L. Qin, R.H. Zhu, Cartilage regeneration using mesenchymal stem cells and a PLGA-gelatin/chondroitin/hyaluronate hybrid scaffold, Biomaterials 27 (2006) 4573–4580.
[9] S. Hudson, E. Magner, J. Cooney, Proteins in mesoporous silicates, Angew. Chem. Int. Edit. 47 (2008) 8582–8594.
[10] M. Adamczak, S.H. Krishna, Enzyme for efficient biocatalysis, Food Technol. Biotechnol. 42 (2004) 251–264.
[11] I.A.M. Ahmed, I. Morishima, E.E. Babiker, N. Mori, Characterisation of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds, Food Chem. 116 (2009) 395–400.
[12] A. Yazgana, G. Özcengiza, E. Özcengizb, K. Kılınçc, M.A. Marahield, N.G. Alaeddinoğlua, Bacilysin biosynthesis by a partially-purified enzyme fraction from *Bacillus subtilis*, Enzyme Microb. Technol. 29 (2001) 400–406.

[13] S.R. Morcelle, C. Liggieri, M. Bruno, N. Priolo, P. Clapés, Screening of plant peptidases for the synthesis of arginine-based surfactants, J. Mol. Catal. B-Enzym. 57 (2009) 177–182.
[14] A. Illanes, Enzyme Biocatalysis: Principles and Applications, first ed., Springer Science, New York, 2008.
[15] D.N. Tran, K.J. Balkus Jr., Perspectives of recent progress in immobilization of enzymes, ACS Catal. 1 (2011) 956–968.
[16] D.K. Eggers, J.S. Valentine, Molecular confinement influences protein structure and enhances thermal protein stability, Protein Sci. 10 (2001) 250–261.
[17] H.-X. Zhou, K.A. Dill, Stabilization of proteins in confined spaces, Biochemistry 40 (2001) 11289–11293.
[18] S. Dumitriu, E. Chornet, Inclusion and release of proteins from polysaccharide-based polyion complexes, Adv. Drug Deliv. Rev. 31 (1998) 223–246.
[19] E.S.N. Kokufuta, H. Tanaka, I. Nakarnura, Use of polyelectrolyte complex-stabilized calcium alginate gel for entrapment of amylase, Biotechnol. Bioeng. 32 (1988) 756–759.
[20] A. Asthana, K.H. Lee, S.-J. Shin, J. Perumal, L. Butler, S.-H. Lee, D.-P. Kim, Bromo-oxidation reaction in enzyme-entrapped alginate hollow microfibers, Biomacrofluidics 5 (2011) 024117–024128.
[21] A. Anwar, S.A.U. Qader, A. Raiz, S. Iqbal, A. Azhar, Calcium alginate: a support material for immobilization of proteases from newly isolated strain of *Bacillus subtilis* KIBGE-HAS, World Appl. Sci. J. 7 (2009) 1281–1286.
[22] M.A.P. Nunes, H. Vila-Real, P.C.B. Fernandes, M.H.L. Ribeiro, Immobilization of naringinase in PVA-alginate matrix using an innovative technique, Appl. Biochem. Biotechnol. 160 (2010) 2129–2147.
[23] E. Quiroga, C.O. Illanes, N.A. Ochoa, S. Barberis, Performance improvement of *araujiain*, a cystein phytoprotease, by immobilization within calcium alginate beads, Process Biochem. 46 (2011) 1029–1034.
[24] M.F. Pardo, M.L.I. López, F. Canals, F.X. Avilés, C.L. Natalucci, N. Caffini, Purification of Balansain I, an endopeptidase from unripe fruits of *Bromelia balansae* Mez (Bromeliaceae), J. Agric. Food Chem. 48 (2000) 3795–3800.
[25] N. Priolo, S. Morcelle del Valle, M. Arribére, L. López, N. Caffini, Isolation and characterization of cysteine protease from the latex of *Araujia hortorum* fruits, J. Protein Chem. 19 (2000) 39–49.
[26] W.D. Obregón, M.C. Arribére, S. Morcelle del Valle, C. Liggieri, N. Caffini, N. Priolo, Two new cysteine endopeptidases obtained from the latex of *Araujia hortorum* fruits, J. Protein Chem. 20 (2001) 317–325.
[27] E. Quiroga, G. Camí, J. Marchese, S. Barberis, Organic solvent effect on the secondary structure of *araujiain* hl, in different media, Biochem. Eng. J. 35 (2007) 1985–2202.
[28] M. Van der Weert, P.I. Haris, W.E. Hennink, D.J.A. Crommelin, Fourier transform infrared spectrometric analysis of protein conformation: effect of sampling methods and stress factors, Anal. Biochem. 297 (2001) 160–169.
[29] D.C. Özturk, D. Kazan, A.A. Denizci, D. Grimoldi, F. Secundo, A. Erarslan, Water miscible mono alcohols effect on the structural conformation of *Bacillus clausii* GMBAE 42 serine alkaline protease, J. Mol. Catal. B-Enzym. 64 (2010) 184–188.
[30] S. Gorinstein, I. Goshev, S. Moncheva, M. Zemser, M. Weisz, A. Caspi, I. Libman, H.T. Lerner, S. Trakhtenberg, O.M. Belloso, Intrinsic tryptophan fluorescence of human serum proteins and related conformational changes, J. Protein Chem. 19 (2000) 637–642.
[31] T. Kijima, S. Yamamoto, H. Kise, Study on tryptophan fluorescence and catalytic activity of α-chymotrypsin in aqueous-organic media, Enzyme Microb. Technol. 18 (1996) 2–6.
[32] F. Şahin, G. Demirel, H. Tümtürk, A novel matrix for the immobilization of acetylcholinesterase, Int. J. Biol. Macromol. 37 (2005) 148–153.
[33] T.S. Pathak, J.-H. Yun, J. Lee, K.-J. Paeng, Effect of calcium ion (cross-linker) concentration on porosity, surface morphology and thermal behavior of calcium alginates prepared from algae (Undaria pinnatifida), Carbohydr. Polym. 81 (2010) 633–639.
[34] M.J. Paquet, M. Laviolette, M. Pézolet, M. Auger, Two-dimensional infrared correlation spectroscopy study of the aggregation of Cytochrome c in the presence of dimyristoylphosphatidylglycerol, Biophys. J. 81 (2001) 305–312.
[35] D. Ajloo, E. Taghizadeha, A.A. Sabouryb, E. Bazyari, K. Mahnamb, Effects of surfactant, salt and solvent on the structure and activity of adenosine deaminase: molecular dynamic and spectrophotometric studies, Int. J. Biol. Macromol. 43 (2008) 151–158.
[36] Y. Liu, X. Zhang, H. Tan, Y. Yan, B.H. Hameed, Effect of pretreatment by different organic solvents on esterification activity and conformation of immobilized *Pseudomonas cepacia* lipase, Process Biochem. 45 (2010) 1176–1180.
[37] S.J. Prestrelski, T. Arakawa, J.F. Carpenter, Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization, Arch. Biochem. Biophys. 303 (1993) 465–473.
[38] J.S. Dordick, Designing enzymes for use in organic solvents, Biotechnol. Prog. 8 (1992) 259–267.
[39] C. Tanford, Chemistry of Macromolecules, Wiley, New York, 1961.
[40] G.E. Schulz, R.H. Schirmer, Principles of Protein Structure, Springer-Verlag, New York, 1979.
[41] K. Ryu, J.S. Dordick, Free energy relationships of substrate and solvent hydrophobicities on enzymatic catalysis in organic media, J. Am. Chem. Soc. 111 (1989) 8026–8027.
[42] A.B. Ross, C. Hall, K. Anastasakis, A. Westwood, J.M. Jones, R.J. Crewe, Influence of cation on the pyrolysis and oxidation of alginates, J. Anal. Appl. Pyrol. 91 (2011) 344–351.
[43] H. Lu, A. Khan, P.G. Smirniotis, Relationship between structural properties and CO_2 capture performance of CaO-based sorbents obtained from different organometallic precursors, Ind. Eng. Chem. Res. 47 (2008) 6216–6220.
[44] R.B. Bhatia, C.J.B. Alok, K. Gupta, A.K. Singh, Aqueous sol–gel process for protein encapsulation, Chem. Mater. 12 (2000) 2434–2441.
[45] S. Kupcu, C. Mader, M. Sara, The crystalline cell surface layer from Thermoanaerobacter thermohydrosulfuricus L111-69 as an immobilization matrix: influence of the morphological properties and the pore size of the matrix on the loss of activity of covalently bound enzymes, Biotech. Appl. Biochem. 21 (1995) 275–286.