ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Technol. Aliment. 22(3) 2023, 361–368
www.food.actapol.net pISSN 1644-0730 eISSN 1898-9594 http://dx.doi.org/10.17306/J.AFS.2023.1171

ORIGINAL PAPER

Received: 29.08.2023
Accepted: 22.10.2023

CHITOSAN FLAKES FOR PAPAIN IMMOBILIZATION: IMPROVING THE STABILITY AND REUSABILITY OF THE ENZYME

Carlos Banchón✉

Environmental Engineering, Escuela Superior Politécnica Agropecuaria de Manabí Manuel Félix López (ESPAM-MFL)
Campus Limón, Calceta 130602, **Ecuador**

ABSTRACT

Background. The global papain market is growing because of its diverse applications in the food, chemical, and textile sectors, although the enzyme's solubility in free form causes reactor losses. This study aims to immobilize papain on chitosan flakes, preserving its catalytic activity for repeated reuse.
Materials and methods. The immobilization process employed citric acid and ethylenediaminetetraacetic acid (EDTA) in a physical adsorption approach.
Results. Papain loading of 0.2 to 0.4 g per gram of chitosan flakes yielded optimal digesting results. When 0.4 g of immobilized papain per gram of chitosan flakes was added, a 2% gelatin solution digested 96.9%.
Conclusion. Notably, papain immobilization on chitosan flakes is achieved without the need for additional chemicals, relying on enzyme concentration and relative enzyme-to-flake weight. This work demonstrates chitosan's potential as an effective medium for physically preserving papain, showcasing its applicability in enzyme immobilization endeavors.

Keywords: physical adsorption, enzyme immobilization, biomaterial, chitin, *Carica papaya* latex, biocatalysis

INTRODUCTION

In today's global chemicals landscape, the focus is squarely on biomolecules like enzymes, renowned for their exceptional catalytic efficiency. Among them, proteases lead the way, comprising 60% of worldwide enzyme sales and finding utility in diverse industries spanning detergents, leather, textiles, food, and pharmaceuticals (Matkawala et al., 2022; Souza et al., 2022). Additionally, plant-derived enzymes such as papain, bromelain, and ficin have carved out their own place in various sectors, from meat tenderization to juice and beer clarification, cosmetics, textiles, baking, cheese production, edema treating, wool antishrinking, amino acid production and beyond (Shin et al., 2020; Benucci et al., 2020; Moreira Filho et al., 2020; Shouket et al., 2020). For instance, papain, sourced from *Carica papaya* latex, serves as a beer clarifier, with an annual consumption of up to 400 tons (Fernández-Lucas et al., 2017). This carbohydrate-free enzyme boasts an alkaline nature (isoelectric point: 9.6), a single polypeptide chain structure (212 amino acids), and a molecular weight of 23,350 Da (He et al., 2010).

The concept of immobilizing enzymes on solid supports or carriers was initially developed to address the pressing problems associated with enzyme recovery and reuse, while simultaneously enhancing the overall biocatalyst quality by providing it with greater stability (Girelli et al., 2020; Bolivar et al., 2022). The immobilization of enzymes offers a range of tangible advantages, like the flexibility to select between

✉carlos.banchon@espam.edu.ec, https://orcid.org/0000-0002-0388-1988

batch or continuous processes, the swift termination of reactions, precise control over product formation, streamlined enzyme removal from reaction mixtures, an augmented resistance to thermal fluctuations, and remarkable adaptability to a wide spectrum of engineered designs (Girelli et al., 2020; Bolivar et al., 2022). In the context of such advancements, the case of papain serves as an illustrative example. To safeguard this proteolytic enzyme from succumbing to auto-proteolysis and to harness its potential more effectively, researchers have adopted the strategic approach of immobilization. This has entailed the use of an array of support materials, including silica spheres, gelatine gels, alginate beads, and chitosan-based matrices (Mosafa et al., 2013; Gu et al., 2018; Zhang et al., 2020; Soares et al., 2020; Lin et al., 2020) characterization, and application of silica-coated magnetic nanoparticles for papain immobilization is reported. Papain was covalently attached onto the (3-chloropropyl).

A primary challenge in enzyme immobilization lies in preventing leakage and ensuring robust bonding between the enzyme and carrier. Various methods have been explored to stabilize papain, such as covalent cross-linking with agents like glutaraldehyde, thionyl chloride, epoxy groups, polyethersulfone, and succinic anhydride, as well as interaction with metal ions (Benucci et al., 2020; Baidamshina et al., 2021; Federsel et al., 2021) novel chitosan–clay nanocomposite films were produced as carriers for the covalent immobilization of papain, by using a fixed amount of chitosan (1% w/v). However, achieving consistent results remains a barrier, and the mechanical properties of the final biocatalyst, determined by the copolymerization process, often cannot be tailored to suit reactor requirements (Husain and Ullah, 2019; Ren et al., 2020; Bolivar et al., 2022). A limitation of covalent binding is the relatively low enzyme quantity immobilized onto carriers (0.02 g per g carrier), hampering widespread industrial use (Maghraby et al., 2023) elevated temperatures, extreme pH values, etc. Consequently, selecting a cost-effective enzyme immobilization approach is imperative to manage operational costs effectively. In this sense, the suitability of chitosan for enzyme immobilization by physical adsorption is underscored by the stability of chitosan particles on surfaces, even amidst variations in pH, ionic strength, and temperature, facilitating effective enzyme immobilization (Aranaz et al., 2021).

Chitosan, a heteropolysaccharide comprising D-glucosamine and N-acetyl-D-glucosamine units, is produced from crustacean shell chitin (Peter et al., 2021). With its notable biodegradability and antimicrobial properties playing a critical role across industries, chitosan finds application in sectors such as food packaging, agriculture, and healthcare; additionally, it is employed in wound care, sutures, drug delivery, and as a component in biodegradable plastics (Girelli et al., 2020; Wang et al., 2020; Kou et al., 2021; Ribeiro et al., 2021). Chitosan's biocompatibility ensures secure interaction with living tissues, promoting plant growth, and providing a protective shield against pathogens and pests (Maluin and Hussein, 2020; Lopez-Nuñez et al., 2022).

The intricacy of immobilizing biocatalysts onto carriers arises from the need for chemical binders to bolster the intricate enzyme-carrier bond. To contribute to our understanding of the use of enzyme technology under mild conditions, this study centers on utilizing chitosan flakes for papain immobilization through chemical binder-free physical adsorption. This research endeavors to amplify papain stability and performance, aiming for extensive industrial applications. Given the limitations concerning enzyme loading onto carriers in papain immobilization approaches, especially issues of leakage and bonding, this study investigates the physical adsorption stabilization technique as a solution. This initiative aligns seamlessly with the overarching objective of optimizing enzyme immobilization, resulting in heightened catalytic efficiency, and paving the way toward expanded biocatalyst utilization across a wide spectrum of industrial sectors.

MATERIALS AND METHODS

Papain extraction and purification

Fresh latex (pH = 6.5) was collected from a five-month-old *C. papaya* fruit using a buffer solution. Papain extraction involved mixing the latex with a 0.1 N buffer solution containing high-purity citric acid (≥99.5%), sodium phosphate (≥99.0%), and EDTA (≥98.0%) in equal proportions (1:1:1), resulting in the extraction of papain mass (in grams). This mix was followed by centrifugation at 3,000 g for 5 min (Yu and Zhang, 2020) the effect of adding different quaternary

ammonium ionic liquids (ILs). In this study, the effect of buffer concentrations ranging from 2.0 to 14.0 mL per gram of *C. papaya* latex on the yield of purified papain was investigated. The resultant supernatant was mixed with a 98° ethanol solution in a ratio of 13:1 (mL per gram of latex), producing a white precipitate. From the optimal buffer concentration, the effect of applying ethanol at application rates ranging from 2.0 to 14.0 mL per gram of latex was evaluated.

Subsequent separation of the precipitate was achieved through centrifugation at 4,000 g and 10°C. The obtained precipitate underwent drying using a freeze dryer (Welch, Germany), and resuspensions were carried out using the 0.1 N citric acid/sodium phosphate/EDTA buffer solution.

The catalytic activity of papain was evaluated by measuring the digestion of casein at 35°C (Arnon, 1970; Moreira Filho et al., 2020; Zhang et al., 2021). The absorbance at 254 nm of undigested casein was compared with the mixture of papain and casein after a 20-min incubation. Equation (1) was then applied to determine the fraction of digestive activity (DA_P).

$$\text{Digestion activity of papain } (DA_P) = \frac{A_{Casein} - A_{Digestion}}{A_{Casein}} \quad (1)$$

Production of chitosan flakes from chitin

Exoskeleton samples of crabs (*Brachyura* spp.) were washed to remove the meat waste and then dried at 40°C for 2 h. They were crushed to a final particle size of between 100 and 400 µm in a hammer mill. To remove the calcium carbonate fraction, 0.10 mL of 1.5 N HCl was added per mg of dried sample and left for 45 min. Demineralized water was used for pH neutralization. To remove the protein fraction, demineralized samples were boiled with a 5% NaOH solution at 100°C for 30 min. Wet solids were deacetylated with a 50% NaOH solution at 100°C for 2 hours (Kou et al., 2021). Precipitates were washed until pH neutralization and thereafter dried at 35°C for about 180 min. Chitosan flakes were obtained by mixing dried chitosan samples with 2% acetic acid at 4°C. The obtained mix underwent freeze drying.

Deacetylation grade

Deacetylation grade (DG) was determined by titration (Ugochukwu et al., 2022). A sample of 500 mg chitosan was added to a flask containing a 100 mL 0.3 N HCl solution. This was titrated with 0.08 N NaOH until the chitosan was totally dissolved. The consumed volume of NaOH (X_1) was computed using Eq. (2). After dissolution, titration continued until this dissolution was gelatinized, and the resulting volume of NaOH is X_2 in the equation below. The pH changes were computed as well.

$$DG = 16.1\ (X_1 - X_2)\frac{N}{m} \quad (2)$$

where

N – normality of NaOH solution

m – amount of chitosan in grams.

Immobilization of papain onto chitosan

A papain solution was added to dried-chitosan flakes at 8°C for 24 h without using any chemicals to bind enzymes and support. In sealed containers, 300 mg of chitosan flakes were mixed with 1 mL of papain solution, yielding concentration ratios of up to 0.4 mg of papain per mg of chitosan. The chitosan flakes were washed with a 0.1 N citric acid/sodium phosphate/EDTA buffer solution until no papain could be detected in the washing solution (Baidamshina et al., 2021) the product of chitin deacetylation, is an excellent candidate for enzyme immobilization purposes. Here we demonstrate that papain, an endolytic cysteine protease (EC: 3.4.22.2). The presence of papain in the washing solution was ascertained through the assessment of its catalytic activity, which was determined using casein as the substrate.

The digestive capacity was assessed by mixing a 2% gelatin solution with wet chitosan containing immobilized papain. The absorbance of the gelatin solution was recorded at 254 nm before and after digestion using a UV-VIS spectrophotometer (Model Genesys 40, Fisher Scientific, USA). The calculation of the fraction of digestion activity of immobilized papain (DA_{ip}) was performed as follows:

$$\text{Digestion activity of imm. papain } (DA_{IP}) = \frac{A_{Gelatin} - A_{Digestion}}{A_{Gelatin}} \quad (3)$$

where

$A_{Gelatin}$ – aborbance at 254 nm of a 2% gelatin solution

$A_{Digestion}$ – aborbance at 254 nm of digested gelatin under different papain concentrations.

All chemicals were purchased from Merck, USA.

Fig. 1. Chitosan flakes

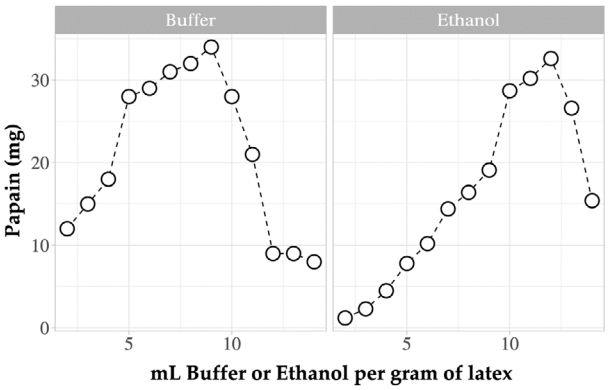

Fig. 2. Amount of purified papain using different application rates of buffer and ethanol

Data analysis

The collected data were analysed by descriptive and inferential statistical methods, employing a significance level of 5%. The effects on digestion activity (response variable) of different amounts of papain and chitosan and the number of times the immobilized papain had been reused were studied using a one-way analysis of variance (ANOVA) within the R-project software environment.

RESULTS AND DISCUSSION

Papain purification and its activity

Figure 2 presents the results elucidating the papain purification process, achieved through the application of an acidic buffer in conjunction with ethanol. The experimental results demonstrate that when employing buffer concentrations ranging from 7.0 to 9.0 mL per gram of latex, a maximum yield of 34 mg of purified papain was attained. Similarly, the utilization of 10 to 12 mL of ethanol yielded up to 32.6 mg of purified dried papain. The addition of ethanol to the extract induces protein denaturation and subsequent aggregation, resulting in their distinct separation from the other components within the mixture. Beyond these specified buffer and ethanol concentrations, the yield of purified papain consistently fell below 2%.

A weight-based analysis conducted in this study indicates that the optimal quantity of purified papain corresponds to a yield of 3.4%, which contrasts with earlier research, where yields of approximately 7% were achieved (Monti et al., 2000; Nitsawang et al., 2006). Papain, being a thiol protease, exhibits the capacity to cleave peptide bonds, specifically those involving aromatic amino acid residues like arginine, lysine, or glutamine in the carbonyl group. This chemical attribute enables the extraction of papain from latex by employing mild acids along with a chelating agent such as EDTA (Yu and Zhang, 2020) the effect of adding different quaternary ammonium ionic liquids (ILs). With regard to pH effects, this study did not detect any significant deviations from the initial acidic pH of 6.5 present in the papaya latex.

Quality control of papain entails assessment of its proteolytic activity, quantified by the acceleration of substrate-to-product conversion due to enzymatic contribution. This catalytic function can also be evaluated in relation to peptide or protein digestion. Figure 3

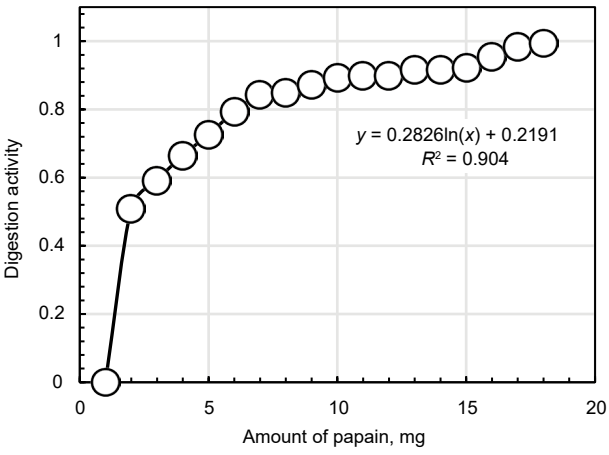

Fig. 3. Digestion activity of different amounts of papain

shows the absorbance data of remaining casein residues, reflecting their digestion efficacy. The optimal concentration range of purified papain solution for efficient milk protein digestion falls within 2.5 to 10 mg per L of solution.

Papain immobilization and its activity

To investigate papain immobilization, varied enzyme quantities were brought into contact with chitosan flakes (DG = 74.88%). The efficacy of these tests was gauged through their impact on gelatin solutions. The enzymatic digestion of gelatin by papain was quantified via changes in absorbance, as shown in Figure 4. Optimal digestion outcomes were achieved within the enzyme loading range of 0.2 to 0.4 g of papain per gram of chitosan flakes. This parameter yielded a remarkable 96.9% digestion effect on a 2% gelatin solution when 0.4 g of immobilized papain was applied per gram of chitosan flakes. However, enzyme quantities falling below 0.15 g of papain per gram of support resulted in digestion percentages below 40%. This assessment underscores the critical enzyme loading requirement for attaining effective gelatin digestion in the immobilization process. In the present study, the absence of reagent addition ensures minimal to negligible conformational changes in papain during immobilization, leading to even distribution of the enzyme across the support through sufficient contact time.

The method of immobilization through physical adsorption is characterized as the most direct technique within the spectrum of immobilization methods, involving the non-covalent binding of enzyme molecules to the support's surface (Baidamshina et al., 2021; Federsel et al., 2021; Wahab et al., 2020) the product of chitin deacetylation, is an excellent candidate for enzyme immobilization purposes. Here we demonstrate that papain, an endolytic cysteine protease (EC: 3.4.22.2). Given the involvement of weak adsorption forces dependent on the support's nature, variables encompassing pH, enzyme and support quantities, time, and temperature require careful control (Rodrigues et al., 2021; Tacias-Pascacio et al., 2021). It is noteworthy that a common misstep in the immobilization process is directly exposing the enzyme to the support without prior pH preconditioning of the support concerning the enzyme solution. Since most carriers are highly reactive materials, side reactions may occur with other components (Itoyama et al., 1994).

Based on the findings presented in Figure 5, this experiment entailed the digestion of a 2% gelatin solution using a catalyst containing 0.4 mg of papain per mg of chitosan over a 1 h period, demonstrating sustained enzymatic activity for up to three cycles of reuse. The more the immobilized enzyme was employed in the gelatin digestion process, the more pronounced

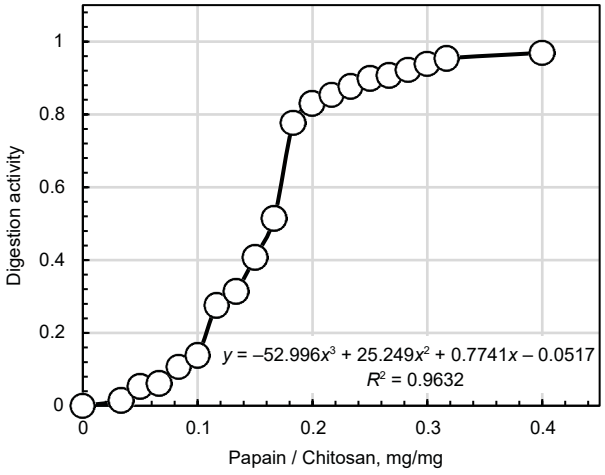

Fig. 4. Digestion activity of different amounts of papain per mg of chitosan flakes

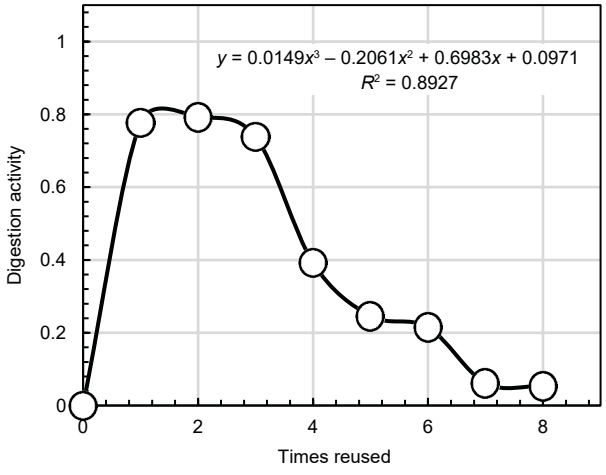

Fig. 5. Digestion activity of immobilized papain after reuse

Table 1. Summary of Descriptive Statistics and F-values from ANOVA for factors affecting digestion activity (*DA*)

Source of variation	*DA* average	*DA* variance	Df	F-value
Mass Papain* DA_P	0.79	0.06	35	35.22 (***)
Ratio Papain/Chitosan* DA_{IP}	0.54	0.15	39	16.68 (***)
Times of reuse* DA_{IP}	0.36	0.11	17	15.64 (**)

Df = Degrees of freedom.
Significance codes: 0 (***); 0.001 (**); 0.01 (*); 0.05 (.); 0.1 (–) no significance (ns).

its loss of activity became. This observation can be attributed to the absence of robust bonding between the support and the enzyme, thereby reducing the probability of effective interaction with active sites (Maghraby et al., 2023) elevated temperatures, extreme pH values, etc. This weakening of interaction is coupled with an elevated probability of entropy increase within the papain-chitosan system, consequently diminishing the spontaneity of catalytic processes.

The underlying mechanism that elucidates the phenomenon of molecular immobilization of papain onto chitosan can be attributed to specific amino acid residues that assist a favorable physical interaction with the polymeric support, as previously discussed (Guisan et al., 2020; Ribeiro et al., 2021). Amino acid residues such as Lys, Asp, Glu, Thr, Ser, Tyr, Cys, and chitosan are involved in the establishment of hydrogen bonds due to their heightened reactivity and propensity for such interactions. Moreover, van der Waals forces contribute to the intricate formation of the novel papain-chitosan system. In a distinct context, the active site of papain encompasses amino acid groups such as Cys and His, which can also engage in bonding with chitosan. This binding of the active site with the support effectively hinders substrate access, thereby negating its catalytic efficacy. However, this occurrence is probabilistic, so that some papain molecules end up with obstructed active sites while others remain unaffected. This duality results in diminished activity.

Significant differences in enzyme activity (referred to as DA_P) can be observed in Table 1, indicating the effects of different papain compositions and quantities of immobilized papain. Moreover, while evaluating the length of carrier utilization, a statistically significant relationship ($p < 0.05$) was observed in relation to digestive activity DA_{IP}. The findings of this study highlight the significance of the quantity of papain and immobilized papain in influencing enzyme function, as well as the differing effects of different operating timeframes on the process of digestion.

CONCLUSIONS

The purification process of papain was achieved under acidic conditions, involving the utilization of citric acid and EDTA as chelating agents. The immobilization of papain through adsorption was influenced by several key factors, including the concentration and quantity of the enzyme in relation to the chitosan flake weight, as well as the ionic strength derived from the concentration of the acidic buffer. Remarkably, chitosan's solubility in acetic acid renders it particularly well-suited for shaping into flake forms, thereby providing an increased surface area for enzyme adsorption. By delving deeper into the intricate mechanisms at play and refining parameters concerning the buffer and ethanol, it may be possible to gain further insights that could improve the efficacy of papain purification processes.

ACKNOWLEDGEMENTS

The author would like to thank Manuel Romo-Leroux for his significant scientific support.

REFERENCES

Aranaz, I., Alcántara, A. R., Civera, M. C., Arias, C., Elorza, B., Heras Caballero, A., Acosta, N. (2021). Chitosan: An Overview of Its Properties and Applications. Polym., 13(19), 3256. https://doi.org/10.3390/polym13193256

Arnon, R. (1970). Papain. In: Methods in Enzymology (pp. 226–244). Vol. 19. Cambridge: Academic Press. https://doi.org/10.1016/0076-6879(70)19017-3

Baidamshina, D. R., Koroleva, V. A., Olshannikova, S. S., Trizna, E. Yu., …, Kayumov, A. R. (2021). Biochemical properties and anti-biofilm activity of chitosan-immobilized papain. Marine Drugs, 19(4), 197. https://doi.org/10.3390/md19040197

Benucci, I., Lombardelli, C., Cacciotti, I., Esti, M. (2020). Papain covalently immobilized on chitosan–clay nanocomposite films: application in synthetic and real white wine. Nanomater., 10(9), 1622. https://doi.org/10.3390/nano10091622

Bolivar, J. M., Woodley, J. M., Fernandez-Lafuente, R. (2022). Is enzyme immobilization a mature discipline? Some critical considerations to capitalize on the benefits of immobilization. Chem. Soc. Rev., 51(15), 6251–6290. https://doi.org/10.1039/D2CS00083K

Federsel, H.-J., Moody, T. S., Taylor, S. J. C. (2021). Recent trends in enzyme immobilization—concepts for expanding the biocatalysis toolbox. Molecules, 26(9), 2822. https://doi.org/10.3390/molecules26092822

Fernández-Lucas, J., Castañeda, D., Hormigo, D. (2017). New trends for a classical enzyme: Papain, a biotechnological success story in the food industry. Trends Food Sci. Technol., 68, 91–101. https://doi.org/10.1016/j.tifs.2017.08.017

Girelli, A. M., Astolfi, M. L., Scuto, F. R. (2020). Agro-industrial wastes as potential carriers for enzyme immobilization: A review. Chemosphere, 244, 125368. https://doi.org/10.1016/j.chemosphere.2019.125368

Gu, Y.-J., Zhu, M.-L., Li, Y.-L., Xiong, C.-H. (2018). Research of a new metal chelating carrier preparation and papain immobilization. Int. J. Biol. Macromol., 112, 1175–1182. https://doi.org/10.1016/j.ijbiomac.2018.02.079

Guisan, J. M., López-Gallego, F., Bolivar, J. M., Rocha-Martín, J., Fernandez-Lorente, G. (2020). The Science of Enzyme Immobilization. In: Guisan, J., Bolivar, J., López-Gallego, F., Rocha-Martín, J. (eds), Immobilization of Enzymes and Cells. Methods in Molecular Biology, vol 2100. New York: Humana. https://doi.org/10.1007/978-1-0716-0215-7_1

He, Z.-Y., Christopher, B.-W., Zhou, Y.-T., Nie, H.-L., Zhu, L.-M. (2010). Papain adsorption on chitosan-coated nylon-based immobilized metal ion (Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}) affinity membranes. Sep. Sci. Technol., 45(4), 525–534. https://doi.org/10.1080/01496390903484784

Husain, Q., Ullah, M. F. (eds.). (2019). Biocatalysis: Enzymatic Basics and Applications. Cham, Switzerland: Springer International Publishing. https://doi.org/10.1007/978-3-030-25023-2

Itoyama, K., Tanibe, H., Hayashi, T., Ikada, Y. (1994). Spacer effects on enzymatic activity of papain immobilized onto porous chitosan beads. Biomater., 15(2), 107–112. https://doi.org/10.1016/0142-9612(94)90258-5

Kou, S. G., Peters, L. M., Mucalo, M. R. (2021). Chitosan: A review of sources and preparation methods. Int. J. Biol. Macromol., 169, 85–94. https://doi.org/10.1016/j.ijbiomac.2020.12.005

Lin, K.-P., Feng, G.-J., Pu, F.-L., Hou, X.-D., Cao, S.-L. (2020). Enhancing the thermostability of papain by immobilizing on deep eutectic solvents-treated chitosan with optimal microporous structure and catalytic microenvironment. Front. Bioeng. Biotechnol., 8, 576266. https://doi.org/10.3389/fbioe.2020.576266

Lopez-Nuñez, R., Suarez-Fernandez, M., Lopez-Moya, F., Lopez-Llorca, L. V. (2022). Chitosan and nematophagous fungi for sustainable management of nematode pests. Front. Fungal Biol., 3, 980341. https://doi.org/10.3389/ffunb.2022.980341

Maghraby, Y. R., El-Shabasy, R. M., Ibrahim, A. H., Azzazy, H. M. E.-S. (2023). Enzyme immobilization technologies and industrial applications. ACS Omega, 8(6), 5184–5196. https://doi.org/10.1021/acsomega.2c07560

Maluin, F. N., Hussein, M. Z. (2020). Chitosan-based agronanochemicals as a sustainable alternative in crop protection. Molecules, 25(7), 1611. https://doi.org/10.3390/molecules25071611

Matkawala, F., Nighojkar, S., Nighojkar, A. (2022). Next-generation nutraceuticals:bioactive peptides from plant proteases. BioTechnologia, 103(4), 397–408. https://doi.org/10.5114/bta.2022.120708

Monti, R., Basilio, C. A., Trevisan, H. C., Contiero, J. (2000). Purification of papain from fresh latex of *Carica papaya*. Braz. Arch. Biol. Technol., 43(5), 501-507. https://doi.org/10.1590/S1516-89132000000500009

Moreira Filho, R. N. F., Vasconcelos, N. F., Andrade, F. K., Rosa, M. D. F., Vieira, R. S. (2020). Papain immobilized on alginate membrane for wound dressing application. Colloids Surf. B: Biointerfaces, 194, 111222. https://doi.org/10.1016/j.colsurfb.2020.111222

Mosafa, L., Moghadam, M., Shahedi, M. (2013). Papain enzyme supported on magnetic nanoparticles: Preparation, characterization and application in the fruit juice clarification. Chinese J. Catal., 34(10), 1897–1904. https://doi.org/10.1016/S1872-2067(12)60663-9

Nitsawang, S., Hatti-Kaul, R., Kanasawud, P. (2006). Purification of papain from *Carica papaya* latex: Aqueous two-phase extraction versus two-step salt precipitation.

Enzyme Microb. Technol., 39(5), 1103–1107. https://doi.org/10.1016/j.enzmictec.2006.02.013

Peter, S., Lyczko, N., Gopakumar, D., Maria, H. J., Nzihou, A., Thomas, S. (2021). Chitin and chitosan based composites for energy and environmental applications: a review. Waste Biomass Valorization, 12(9), 4777–4804. https://doi.org/10.1007/s12649-020-01244-6

Ren, D., Wang, Z., Jiang, S., Yu, H., Zhang, S., Zhang, X. (2020). Recent environmental applications of and development prospects for immobilized laccase: A review. Biotechnol. Genet. Eng. Rev., 36(2), 81–131. https://doi.org/10.1080/02648725.2020.1864187

Ribeiro, E. S., De Farias, B. S., Sant'Anna Cadaval Junior, T. R., De Almeida Pinto, L. A., Diaz, P. S. (2021). Chitosan–based nanofibers for enzyme immobilization. Int. J. Biol. Macromol., 183, 1959–1970. https://doi.org/10.1016/j.ijbiomac.2021.05.214

Rodrigues, R. C., Berenguer-Murcia, Á., Carballares, D., Morellon-Sterling, R., Fernandez-Lafuente, R. (2021). Stabilization of enzymes via immobilization: Multipoint covalent attachment and other stabilization strategies. Biotechnol. Adv., 52, 107821. https://doi.org/10.1016/j.biotechadv.2021.107821

Shin, D., Mukherjee, R., Grewe, D., Bojkova, D., Baek, K., …, Dikic, I. (2020). Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity. Nature, 587(7835), 657–662. https://doi.org/10.1038/s41586-020-2601-5

Shouket, H. A., Ameen, I., Tursunov, O., Kholikova, K., Pirimov, O., …, Mukimov, B. (2020). Study on industrial applications of papain: A succinct review. IOP Conference Series: Earth and Environmental Science, 614(1), 012171. https://doi.org/10.1088/1755-1315/614/1/012171

Soares, A. M. B. F., Gonçalves, L. M. O., Ferreira, R. D. S., De Souza, J. M., Fangueiro, R., …, Cantanhêde, W. (2020). Immobilization of papain enzyme on a hybrid support containing zinc oxide nanoparticles and chitosan for clinical applications. Carbohydr. Polym., 243, 116498. https://doi.org/10.1016/j.carbpol.2020.116498

Souza, T. F., Martins, M. L., Magno, M. B., Vicente-Gomila, J. M., Fonseca-Gonçalves, A., Maia, L. C. (2022). Worldwide research trends on the use of chemical–mechanical caries removal products over the years: A critical review. Eur. Arch. Paediatr. Dent., 23(6), 869–883. https://doi.org/10.1007/s40368-022-00726-6

Tacias-Pascacio, V. G., Morellon-Sterling, R., Castañeda-Valbuena, D., Berenguer-Murcia, Á., Kamli, M. R., Tavano, O., Fernandez-Lafuente, R. (2021). Immobilization of papain: A review. Int. J. Biol. Macromol., 188, 94–113. https://doi.org/10.1016/j.ijbiomac.2021.08.016

Ugochukwu, M., Okwulehie, I., Egbucha, K. (2022). Chitosan production of *Pleurotus ostreatus* (Jacq. Ex fr.) p. kumm cultivated on different substrates. Int. J. Sci. Appl. Res., 29(2).

Wahab, R. A., Elias, N., Abdullah, F., Ghoshal, S. K. (2020). On the taught new tricks of enzymes immobilization: An all-inclusive overview. React. Funct. Polym., 152, 104613. https://doi.org/10.1016/j.reactfunctpolym.2020.104613

Wang, W., Meng, Q., Li, Q., Liu, J., Zhou, M., Jin, Z., Zhao, K. (2020). Chitosan derivatives and their application in biomedicine. Int. J. Molec. Sci., 21(2), 487. https://doi.org/10.3390/ijms21020487

Yu, L., Zhang, H. (2020). Separation and purification of papain crude extract from papaya latex using quaternary ammonium ionic liquids as adjuvants in PEG-Based aqueous two-phase systems. Food Anal. Methods, 13(7), 1462–1474. https://doi.org/10.1007/s12161-020-01761-z

Zhang, X., Shuai, Y., Tao, H., Li, C., He, L. (2021). Novel method for the quantitative analysis of protease activity: the casein plate method and its applications. ACS Omega, 6(5), 3675–3680. https://doi.org/10.1021/acsomega.0c05192

Zhang, Z., Bai, G., Xu, D., Cao, Y. (2020). Effects of ultrasound on the kinetics and thermodynamics properties of papain entrapped in modified gelatin. Food Hydrocoll., 105, 105757. https://doi.org/10.1016/j.foodhyd.2020.105757