I COBBIND
I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM ·· XV SHEB ·· XV ENZITEC FLORIANÓPOLIS ·· 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil

IMMOBILIZATION OF TRYPSIN ON A NEW SUPPORT FROM CHITOSAN HYDROGEL – EFFECT OF pH ON THE ADSORPTION PROCESS

João P. Miguez[1], Adriano A. Mendes[1]*

[1] *Institute of Chemistry, Federal University of Alfenas, Alfenas – MG, Brazil.*
* *Corresponding author's email address: adriano.mendes@unifal-mg.edu.br*

ABSTRACT

The aim of this new study was to develop an active biocatalyst via physical adsorption of trypsin from porcine pancreas on a heterofunctional support prepared by sequential activation of chitosan hydrogel (Chit) with glutaraldehyde (GA) and functionalization with glycine (Chit–GA–Gly). The catalytic performance of the biocatalyst in hydrolysis reaction was compared with the immobilized enzyme on classical glutaraldehyde-activated chitosan (Chit–GA) and chitosan hydrogel (Chit) without chemical modification. The highest immobilized protein loading on the new support Chit–GA–Gly was around of 16 mg·g^{-1} at pH 9.0 (5 mmol·L^{-1} buffer sodium carbonate) at 25 °C using an initial protein loading of 20 mg·g^{-1}. This biocatalyst exhibited a maximum specific activity (SA) of 33.1 ± 0.2 nmol·mg^{-1} for benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) hydrolysis, twice as high as the enzyme immobilized on the classic Chit–GA support (SA values ranging between 6.7 ± 0.1 nmol·mg^{-1} and 8.1 ± 0.1 nmol·mg^{-1}). These results indicate that this new heterofunctional support can be used as an interesting alternative for preparing biocatalysts with high catalytic activity for production of valuable compounds such as protein hydrolysates.

Keywords: Trypsin. Porcine Pancreas. Immobilization. Heterofunctional support. Chitosan.

1 INTRODUCTION

Trypsin (EC 3.4.21.4) is a serine protease from porcine pancreas and serves as one of the primary digestive enzymes secreted by the pancreas of animals. Its vital role in the digestive process also involves the activation of zymogens, namely chymotrypsinogen and procarboxypeptidase.[1] This hydrolytic enzyme has been industrially used in leather processing, proteome analysis, food protein allergies reduction, bioactive peptides production, and preparation of digestible dairy products for infants.[2] However, free trypsin exhibits low stability under specific process conditions (high temperatures, presence of organic solvents, and extreme pH conditions) along with challenges in separating the enzyme from the final product, thus hindering its recovery and subsequent reuse.[3,4] In order to overcome such limitations, different enzyme immobilization protocols have been proposed to allow for the repeated use of the enzyme, thereby facilitating efficient recovery from reaction mediums, simplifying separation processes, and reducing contamination risks in both the enzymes and final products.[2] Immobilized enzymes, compared to their soluble form, may show enhanced stability if properly performed, increasing the operation window.[2–4] Notably, trypsin immobilization inside porous supports drastically minimizes the autolysis process in proteases like trypsin.[5,6]

In this context, the objective of this study was to prepare a heterofunctional support (Chit-GA-Gly) from chitosan, a natural polysaccharide obtained by deacetylation of chitin from various sources, including the exoskeleton of insects and crustaceans like crabs, shrimp, and lobsters.[7] This new support was prepared via first activation of chitosan hydrogels (Chit) with glutaraldehyde (GA), followed by functionalization with glycine (Gly). It has cationic groups from the support and Gly (pre-existing and introduced protonated amino groups), anionic groups from the Gly (introduced carboxylate groups), and some hydrophobicity from glutaraldehyde moieties.[8] The influence of pH on immobilization process and properties of the biocatalyst was evaluated and its performance was compared with chitosan hidrogels without chemical modification (Chit) and the conventional chitosan hydrogel activated with glutaraldehyde (Chit–GA).

2 MATERIAL & METHODS

2.1. Materials – Trypsin from porcine pancreas (T-0303, Type IX-S, lyophilized, 13,000-20,000 BAEE units.mg^{-1} protein), and benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Shrimp chitosan (powder form, with a 75-85% degree of deacetylation and a molecular mass distribution between 50 and 190 kDa) was also obtained from Sigma-Aldrich. Glutaraldehyde solution at 25% v.v^{-1} and glycine (Gly) were purchased from Synth® (São Paulo, SP, Brazil). All other chemical reagents and organic solvents were of analytical grade from Synth®.

2.2. Preparation of the supports – Glutaraldehyde-activated chitosan hydrogel (Chit–GA) was prepared according to a previous study.[9] 5 g of powdered chitosan were added to 100 mL of a glacial acetic acid solution (5% v.v^{-1}) at 25 °C for 4 h under continuous mechanical stirring at 1000 rpm. The solution was then introduced via syringe into a 0.1 mol.L^{-1} NaOH solution under mild mechanical stirring (100 rpm) at 25 °C for 12 h to produce the chitosan hydrogel (Chit). The resulting suspension was filtered, washed with distilled water, until pH 7.0, and stored at 4 °C. Then, 10 g of chitosan hydrogel (Chit) were subsequently immersed in a fresh glutaraldehyde solution, accomplished by adding 11.2 mL of 0.2 mol.L^{-1} sodium phosphate solution at pH 7.0 to 16.8 mL of a 25% glutaraldehyde solution (v.v^{-1}). In this step, GA molecules react preferentially with pre-existing free amine groups of chitosan polymer to form stable imine bonds. The suspension was subjected to continuous mechanical stirring (200 rpm) at 25 °C for 1 h to yield the glutaraldehyde-activated chitosan hydrogel (Chit–GA). This activated support was also filtered, washed with distilled water, until pH 7.0, and stored at 4 °C. The heterofunctional support (Chit–GA–Gly) was prepared by suspending 10 g of

Chit–GA in 90 mL of a 0.5 mol.L^{-1} glycine solution at pH 8.0, at 25 °C under orbital agitation (200 rpm) for 48 h. In this step, glycine reacts with aldehyde groups of Chit–GA to produce a heterogeneous matrix with high density of protonated imine (cationic) and carboxylate (anionic) groups. The scheme of preparation and functionalization of this heterofunctional support is shown in a previous study performed in our group. [8]

2.3. General immobilization procedure of trypsin on the different supports – 10 g of each support (Chit, Chit–GA and Chit–GA–Gly) were added to 190 mL of trypsin solution prepared at 5 mmol L^{-1} buffer solutions using a fixed initial protein loading of 20 protein mg g^{-1} of support. The suspensions were added to a 300 mL closed flasks and immersed in a temperature-controlled water-bath under continuous stirring (200 rpm) at 25 ± 1 °C for 15 h. The immobilized biocatalysts were recovered via filtration and washed with distilled water. The adsorption process was monitored by measuring the residual protein concentration.[10] The hydrolytic activity (HA) of soluble and immobilized enzyme was determined by using BAPNA as substrate (919.7 µmol·L^{-1}) at pH 8.0 (50 mmol·L-1 sodium phosphate) at 37 °C under constant stirring (200 rpm).[11] The reaction was monitored spectrophotometrically at 410 nm. The enzymatic activity was calculated using a constructed analytical curve of p-nitroaniline (the released product after BAPNA hydrolysis). The activity was expressed in nmol·g^{-1} (for immobilized enzyme) and nmol·mL^{-1} (for soluble enzyme). The immobilization yield percentage (IY) was calculated based on the hydrolytic activity units disappeared in the supernatant solution.[12] Specific activity (SA – $U.mg_{protein}^{-1}$) values were determined as being the ratio between hydrolytic activity and protein concentration.[8]

3 RESULTS & DISCUSSION

According to results summarized in Table 1, the adsorption capacity of the newly prepared support (Chit–GA–Gly) increased with an increase in pH solution. This fit with the high isoelectric point of trypsin (between 10.1 and 10.5),[11] the protein will mainly have a cationic character at pH lower than 10. In acidic conditions (pH between 4.0 and 6.0), The lowest amount of immobilized protein, ranging from 5.4 ± 0.3 to 6.8 ± 0.1 mg enzyme·g^{-1} of support, was observed. In a neutral medium, an increase in the amount of immobilized protein may be observed, around of 9 mg·g^{-1} of support (IY=44.5%). When the pH of the solution was raised from 7.0 to 9.0 (decreasing the cationic character of the enzyme but simultaneously decreasing the cationic character of the chitosan and of the amino groups of Gly, but remarking the anionic character of the carboxylate groups of Gly), the concentration of immobilized protein almost doubled, increasing from 9.1 ± 0.3 mg·g^{-1} (pH 7.0) to 16.2 ± 0.2 mg·g^{-1} (pH 9.0), thus resulting in a higher IY – 81% (see Table 1).

Table 1 Influence of pH solution on the immobilization parameters of trypsin from porcine pancreas in Chitosan (Chit), Chitosan-Glutaraldehyde (Chit–GA) and Chitosan-Glutaraldehyde-Glycine (Chit–GA–Gly) hydrogels.

Support	pH	IP[a] (mg.g^{-1})	IY[b] (%)	HA[c] (nmol.g^{-1})	SA[d] (nmol.mg^{-1})
Chit–GA–Gly	4.0	6.8 ± 0.2	30.7 ± 0.5	18.1 ± 2.0	2.7 ± 0.3
	5.0	5.4 ± 0.3	25.6 ± 1.1	29.7 ± 0.8	4.4 ± 0.1
	6.0	6.8 ± 0.1	32.4 ± 0.5	45.5 ± 0.9	6.7 ± 0.1
	7.0	9.1 ± 0.3	44.5 ± 1.1	115.5 ± 3.7	17.3 ± 0.5
	8.0	11.7 ± 0	56.2 ± 0.2	163.3 ± 2.4	23.8± 0.4
	9.0	16.2 ± 0.2	81.0 ± 0.2	224.8 ± 1.6	33.1 ± 0.2
	10.0	15.1 ± 0.1	71.6 ± 0.2	117.2 ± 5.9	26.1 ± 0.9
Chit	8.0	2.1 ± 0.4	10.5±1.8	57.6 ± 5.7	26.9 ± 2.7
	9.0	3.8 ± 0.3	19.8 ± 1.4	84.3 ± 2.6	21.9 ± 0.7
Chit–GA	7.0	20.0	100.0	161.5 ± 1.1	8.1 ± 0.1
	8.0	20.0	100.0	139.3 ± 2.2	7.1 ± 0.2
	9.0	19.1 ± 0.2	98.6 ± 0.8	129.1 ± 2.1	6.7 ± 0.1

a - Immobilized protein concentration; b – Immobilized yield; c - Hydrolytic activity; d - Specific activity;

Likewise, the highest values of hydrolytic activity (HA = 224.8 ± 1.6 nmol·g^{-1}) and specific activity (SA = 33.1 ± 0.2 nmol·mg^{-1} of immobilized protein) were also observed at pH 9.0. These results clearly demonstrate the strong influence of ionic character on the enzyme and support surfaces during the immobilization process. Therefore, adsorption by ionic interaction is one of the mechanisms involved in the immobilization process, consistent with a previous study on the immobilization of lipase from *Thermomyces lanuginosus* (TLL) using this heterofunctional support.[8] At pH values below 10.1, the enzyme becomes positively charged. Consequently, the interaction of the enzyme occurs through positively charged groups on the surface (protonated amino groups of the lysine, arginine, and terminal amino residues) with the carboxylate groups introduced by functionalization with Gly. Immobilization at pH higher than the support pI gives the support a negative charge (due to the increased density of negative charges from the deprotonation of the carboxylate groups introduced by functionalization with Gly). Therefore, the interaction between the enzyme and the support is influenced by preferential interactions between these groups and the protonated amino groups on the enzyme's surface. At pH 9.0, the medium in which the support exhibited the highest adsorption capacity and catalytic performance (expressed in terms of HA and SA values), the interaction between enzyme and support may involve these interactions mentioned above. Additionally, other ionic interactions may occur between the deprotonated carboxylate groups of aspartate and glutamate residues, as well as the carboxy-terminal group, with protonated amino groups on the support of chitosan introduced by functionalization with Gly, further contributing to the immobilization process. It should be noted that the immobilization of enzymes via ionic interactions is a heterogeneous and multipoint process involving various ionic groups of the enzyme and the support,[12] even of different nature.[13]

Moreover, hydrophobic interactions generated by the presence of glutaraldehyde molecules also seem to play a critical role to the immobilization process, as the maximum adsorption capacity in not in the middle of the pI of support and enzyme.[8] This indicates that the interaction between the enzyme and the prepared support is a complex process involving different types of interactions.

The chitosan hydrogel (Chit) without chemical modification, that is just an anionic exchanger, exhibited a relatively low immobilization capacity within the optimum pH range of 8.0 to 9.0, yielding less than 4 $mg \cdot g^{-1}$ (or an IY less than 20%), as the protein will be a multi-cation polymer under these conditions. Moreover, the hydrolytic activity values were comparably lower, ranging from 57.6 ± 5.7 $nmol \cdot g^{-1}$ at pH 8.0 to 87.3 ± 2.6 $nmol \cdot g^{-1}$ at pH 9.0, compared to the hydrolytic activity values obtained when using Chit–GA–Gly. By contrast, the specific activity values, varying from 26.9 ± 2.7 $nmol \cdot mg^{-1}$ at pH 8.0 to 21.9 ± 0.7 $nmol \cdot mg^{-1}$ at pH 9.0, were higher than those obtained with the immobilized enzyme on the new support, which can be attributed to lower limitations for substrate diffusion, the treatment with glutaraldehyde can reduce the pore diameter. Moreover, as the enzyme loading is smaller, the effects of the substrate diffusion limitations are smaller.

Trypsin was fully immobilized on Chit–GA within the optimum pH range of 7.0 to 9.0. The immobilization process in this support is attributed to first ion exchange/hydrophobic interactions, followed by the formation of covalent bonds between enzyme reactive groups and the glutaraldehyde groups introduced into the support.[8] Under these experimental conditions, we observed maximum hydrolytic activity at 161.5 ± 1.1 $nmol \cdot g^{-1}$ for pH 7.0. Regarding specific activity, these values were almost two times lower than those obtained for the new support (Chit–GA–Gly). Consequently, the enzyme immobilization pH at pH 9.0 on Chit–GA–Gly provides a biocatalyst with highest enzyme retention capacity and catalytic performance.

4 CONCLUSION

This study clearly showed that the new heterogeneous support prepared in this study containing high density of cationic (protonated imine) and anionic (carboxylate) groups in its surface can be successfully used in the preparation of industrial biocatalysts via ionic interactions due to its high protein capacity and catalytic activity. The development of an efficient and sustainable support from renewable materials can be considered a promising field for environmental researchers. Further studies can be conducted using the resulting protein hydrolysates, including bioactivity tests to explore their antioxidant, antimicrobial, antihypertensive, and anti-inflammatory properties.

REFERENCES

[1] NAVEED, M.; NADEEM, F.; MEHMOOD, T.; BILAL, M.; ANWAR, Z.; AMJAD, F. 2021. Catal Lett. 151 (2) 307-323.
[2] TAVANO, O.L.; BERENGUER-MURCIA, A.; SECUNDO, F.; FERNANDEZ-LAFUENTE, R. 2018. Compr. Rev. Food Sci. Food Saf. 17 (1) 412-436.
[3] BOLIVAR, J.M.; WOODLEY, J.M.; FERNANDEZ-LAFUENTE, R. 2022. Chem Soc Rev 51 (7) 6251-6290.
[4] MAGHRABY, Y.R.; EL-SHABASY, R.M.; IBRAHIM, A.H.; AZZAZY, H.M.E.S. 2023. ACS Omega 8 (8) 5184-5196.
[5] MASSOLINI, G.; CALLERI, E. 2005. J. Sep. Sci. 28 (1) 7-21.
[6] SASAI, Y.; KANNO, H.; DOI, N.; YAMAUCHI, Y.; KUZUYA, M.; KONDO, S.I. 2017. Catalysts 7 (1) 4.
[7] KOU, S.; PETERS, L.M.; MUCALO, M.R. 2021. Int. J. Biol. Macromol. 169 (1) 85-94.
[8] OKURA, N.S.; SABI, G.J.; CRIVELLENTI, M.C.; GOMES, R.A.B.; FERNANDEZ-LAFUENTE, R.; MENDES, A.A. 2020. Int. J. Biol. Macromol. 163 (1) 550-561.
[9] MENDES, A.A.; CASTRO, H.F.; RODRIGUES, D.S.; ADRIANO, W.S.; TARDIOLI, P.W.; MAMMARELLA, E.J.; GIORDANO, R.C.; GIORDANO, R.L.C. 2011. J. Ind. Microbiol. Biotechnol. 38 (2) 1055-1066.
[10] PESSATO, T.B.; TAVANO, O.L. 2015. Acta Aliment. 44 (2) 570-577.
[11] BRADFORD, M.M. 1976. Anal. Biochem. 72 (1) 248-254.
[12] BOUDRANT, J.; WOODLEY, J.M.; FERNANDEZ-LAFUENTE, R. 2020. Process Biochem. 90 (1) 66-80.
[13] SOTO, I.D.; ESCOBAR, S.; MESA, M. 2017. Mater. Sci. Eng. C 79 (1) 525-532.

ACKNOWLEDGEMENTS

The present study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Brazil – Finance Code 001. The authors are also thankful for the financial support of Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) – Brazil (Process APQ-01691-21). Adriano A. Mendes thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the research fellowship (PQ 2 – CA BI – Grant 306253/2023-2).