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IMMOBILIZATION OF HORSODE PEROXIDASE BY TRAPPING AND COVALENT BONDING IN ALGINATE-PECTIN AND ALGINATE-PECTIN-STARCH HYBRID SUPPORT

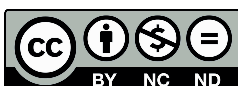
Ani Caroline Weber
Universidade do Vale do Taquari
Post-graduation Program in Biotechnology
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/8238624023188773

Bruno Eduardo da Silva
Universidade do Vale do Taquari
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/9474409267081328

Joana Elisa Willrich
Universidade do Vale do Taquari
Lajeado - Rio Grande do Sul
https://lattes.cnpq.br/3752000520359166

Bruna Costa
Universidade do Vale do Taquari
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/2255574648252180

Cristiano de Aguiar Pereira
Universidade do Vale do Taquari
Post-graduation Program in Biotechnology
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/4370455437960177



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Giovana Schneider
Universidade do Vale do Taquari
Post-graduation Program in Biotechnology
Lajeado - Rio Grande do Sul
https://lattes.cnpq.br/4269473693605873

Sabrina Grando Cordeiro
Universidade do Vale do Taquari
Post-graduation Program in Biotechnology
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/2337098282279202

Daniel Augusto Weber
Universidade do Vale do Taquari
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/6003642280026432

Jéssica Samara Herek dos Santos
Universidade do Vale do Taquari
Post-graduation Program in Biotechnology
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/8038392775385994

Eduardo Miranda Ethur
Universidade do Vale do Taquari
Post-graduation Program in Biotechnology
Post-graduation Program in Environment and Development
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/0536800052883688

Elisete Maria de Freitas
Universidade do Vale do Taquari
Post-graduation Program in Biotechnology
Lajeado - Rio Grande do Sul
http://lattes.cnpq.br/7345668866571738

Filipa Rego Pinto Gomes
Instituto Politécnico de Leiria – IPLeiria
Leiria - Portugal
https://www.cienciavitae.pt/6F1C-0BAD-DC72

Lucélia Hoehne
Universidade do Vale do Taquari
Post-graduation Program in Biotechnology
Post-graduation Program in Environment and Development
Lajeado – Rio Grande do Sul
http://lattes.cnpq.br/1088266827926373

Abstract: Horseradish peroxidase (HRP) is an enzyme with excellent biocatalytic activity and potential for industrial application. Aiming at large-scale applications, enzyme immobilization appears as an alternative for recovering and reusing enzymes, enabling employability and reducing costs. In this sense, the use of biopolymers has proven to be interesting, as they are non-toxic, biodegradable, renewable and highly available. However, there is still a lack of studies aimed at immobilizing HRP in hybrid biopolymers containing alginate, pectin and/or starch. Therefore, the present study aimed to immobilize HRP, through entrapment and covalent bonding, in alginate-pectin and alginate-pectin-starch granules, in addition to a control (alginate). For this, different gel compositions were prepared and extruded in calcium chloride solution using a syringe and needle (Ø = 1 mm). The granules were cross-linked by inserting them into a 0.02% glutaraldehyde solution. The parameters yield, efficiency and activity recovery were evaluated at time 0 h (initial) and after seven days of storage. The immobilization of HRP on alginate-pectin-starch granules proved to be superior in relation to alginate-pectin granules when evaluating the set of parameters, resulting in an activity recovery of 11.21% at time 0 h and 10.51% after seven days of storage. However, it is important to highlight that the control formulation (alginate), among all, showed the best recovery of activity after the storage period. Thus, in general, it can be concluded that the addition of pectin and/or starch in the composition of hybrid alginate gels has potential, however, further studies and optimizations are necessary.

Keywords: Horseradish peroxidase. *Armoracia rusticana*. Biopolymer. Hybrid support.

INTRODUCTION

Horseradish peroxidase (Horseradish peroxidase - HRP) is an enzyme belonging to the peroxidase family, obtained from the roots of the Armoracia rusticana species. HRP has efficient biocatalytic activity, being an ecological biocatalyst widely studied due to the constant increase in its use in industries, since enzyme availability is low cost and extraction and purification are relatively easy (LENG et al., 2020). However, for large-scale applications, some obstacles are observed in the use of the enzyme in its free form, such as the impossibility of recovery and reuse, since HRP is soluble in water, in addition to low stability over time. time (LI et al., 2019). Seeking to solve these problems, enzyme immobilization has proven to be a promising alternative.

Enzymatic immobilization corresponds to the process of converting an enzyme from its soluble form to an insoluble form, commonly carried out through enzymatic binding to a solid material and which allows, in many cases, the obtaining of a stable biocatalyst with catalytic activity superior to that of of the free enzyme, capable of being reused for several subsequent cycles (PETRONIJEVIĆ et al., 2021). Several immobilization techniques are known and used, such as entrapment and covalent bonding. When covalently immobilized, the enzyme is strongly bound to the support, minimizing leaching and enzyme leakage, preventing protein contamination of the product (ZAHIRINEJAD et al., 2021). In the entrapment method, the enzyme is trapped in a polymeric network, providing it with protection due to the absence of direct contact with the external environment, improving stability and reducing the risk of denaturation and leaching (RIBEIRO et al., 2021).

For immobilization, numerous supports can be used, such as biopolymers. The

immobilization of enzymes in biopolymers is well established and has been the subject of numerous studies, as biomaterials are attractive due to their non-toxicity, biodegradability, flexibility and availability. Among the most promising materials for enzyme immobilization are those based on carbohydrates, such as alginate, cellulose, chitosan, chitin and agarose (IMAM; MARR P.; MARR A., 2021). Alginate is a negatively charged polysaccharide, composed of two isomer residues (β-D-mannuronic acid and α-L-guluronic acid) linked by glycosidic bonds (1 → 4), which in the presence of divalent metal ions cross-link, forming a biopolymer gel (CHENG et al., 2020). For enzymatic immobilization, alginate can be combined with other materials, forming hybrid supports (IMAM; MARR P.; MARR A., 2021), with greater mechanical resistance and less enzymatic leaching during application (BILAL; IQBAL, 2019).

In this sense, some biopolymers that emerge as an interesting alternative for combining with alginate are starch and pectin. Starch is a natural polysaccharide, produced by plants as energy storage, being extensively used in food and non-food industries due to its non-toxicity, low cost, wide accessibility, biocompatibility and biodegradability (SHOKRI et al., 2022). Pectins are a group of closely related complex polysaccharides, being structural constituents of primary and secondary cell walls and middle lamellae in plant tissues. Due to their excellent gelling properties and cytocompatibility, pectins have been studied in biomedical and pharmaceutical applications, as well as in enzyme immobilization, as they are biocompatible, biodegradable, low-cost, non-toxic and renewable (BILAL; IQBAL, 2019; NEMIWAL; ZHANG; KUMAR, 2021).

Although alginate, starch and pectin have been demonstrated as potential alternatives for enzymatic immobilization, few studies involving the combination of these biopolymers to form a hybrid support for HRP immobilization have been found. Therefore, this study aimed to evaluate the immobilization of commercial HRP by entrapment and covalent bonding on an alginate-pectin and alginate-pectin-starch hybrid support.

MATERIALS AND METHODS

CHEMICAL MATERIALS/REAGENTS

The Horseradish peroxidase (Horseradish peroxidase - HRP, CAS 9003-99-0, 77332, lyophilized powder, 40 kDa, 173 U/mg), bovine serum albumin (CAS 9048-46-8, A2153, lyophilized powder, 66 kDa, ≥96%) and glutaraldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Food grade sodium alginate was purchased from GastronomyLab (Distrito Federal, Brazil), P.A. potato starch from Cinética (São Paulo, Brazil), food grade pectin from Mago (São Paulo, Brazil), anhydrous dibasic sodium phosphate P.A. (Na_2HPO_4) and anhydrous calcium chloride P.A. from Vetec Química (Rio de Janeiro, Brazil), ortho-phosphoric acid (H_3PO_4) 85% P.A. from Nuclear (SP, Brazil), sodium hydroxide (NaOH) P.A. from Êxodo Científica (SP, Brazil), guaiacol

($C_7H_8O_2$) from Neon (SP, Brazil), hydrogen peroxide 30% (H_2O_2) from ``Química Moderna`` (SP, Brazil) and the Bradford dye from Bio-Rad (CA, USA).

PROTEIN QUANTIFICATION

Protein quantification was performed using the Bradford method (1976), using bovine serum albumin (BSA) as a protein standard. The calibration curve was constructed using BSA concentrations equivalent to 0; 0.2; 0.4; 0.6; 0.8 and 1.0 mg/mL. To do this, 4 μL of BSA solution of each concentration (or sample) in quintuplicate and 200 μL of Bradford dye (Coomassie brilliant blue G250) were

added to the 96-well plate. Wait 10 minutes and read the absorbance at 595 nm, using a Spectramax microplate reader (Spectramax i3, Molecular Devices, CA, USA). Using the calibration curve and the absorbance of the samples obtained, the protein concentration was calculated.

ENZYMATIC ACTIVITY

The enzymatic activity of free or immobilized HRP was determined according to the methodology adapted from Queiroz et al. (2018), using guaiacol as a substrate. Thus, in a cuvette were added 2700 μL of 0.1 M sodium phosphate buffer (pH 6.0), 100 μL of 0.01 M hydrogen peroxide (H2O2), 100 μL of 0.1 M guaiacol and 100 μL of enzyme solution or 0.1 g of derivative (support + immobilized enzyme). After 1 min of reaction, the absorbance was read using a Molecular Absorption Spectrophotometer in the Ultraviolet-Visible (UV-Vis) region (Genesys 10S, Thermo Scientific, USA), at 470 nm. For the blank, the enzyme solution was replaced by sodium phosphate buffer (free HRP) or support beads without the presence of enzyme (immobilized HRP). Using the absorbances obtained and using Equation 1, the enzymatic activity was calculated. One unit of enzymatic activity (U) corresponds to the amount of enzyme necessary to catalyze the transformation of 1 μmol of guaiacol per minute.

$$A.E.(U/mL) = \frac{(Abs\ (t) - Abs\ (0)) * V_{total} * 1000}{\varepsilon * V_{enzyme} * t}$$

(Equation 1)

Where Abs (t) is the absorbance verified after 1 minute of reaction, Abs (0) the absorbance over time: 0, V_{total}, the total volume in the cuvette (3 mL), ε the molar absorptivity coefficient of guaiacol (26600 $L.mol^{-1}.cm^{-1}$), V_{enzyme}, the volume (mL) of enzyme solution added to the cuvette and t the time (min) elapsed.

ENZYME IMMOBILIZATION

Enzymatic immobilization was performed according to the methodology adapted from Bilal et al. (2016). Two hybrid gel formulations were prepared (alginate-pectin and alginate-pectin-starch), in addition to a formulation containing only alginate (control) and blank (without enzyme), as shown in Table 1.

For all gels, a total volume of 25 mL was prepared, so all reagents were added considering this final volume. All steps were carried out at room temperature (22 ± 2°C), except when values other than this were described. Thus, appropriate amounts of sodium alginate, starch and/or pectin were added to 20 mL of ultrapure water (18.2 MΩ.cm-1, Milli-Q, Millipore, USA), and stirred on a magnetic plate. until complete homogenization of the gel (no lumps). Then, an appropriate amount of HRP enzymatic solution previously prepared in 0.1 M phosphate buffer (pH 6.0) (with an activity of 80.2 U/mL) was inserted. It was stirred on a magnetic plate for 15 minutes, 50% (v/v) glutaraldehyde was added to obtain a concentration of 0.02% (v/v) in the gel, and the total volume was completed until 25 mL with ultrapure water. It was homogenized for 2 hours on a magnetic plate and the gel was left to rest at 4 °C for 15 hours to eliminate bubbles.

The gel obtained for each formulation was extruded separately, using a syringe and needle (diameter equal to 1 mm), in 150 mL of 0.2 M calcium chloride (CaCl2) solution under mild magnetic stirring and maintained at 4 ° C using an ice bath. The granules were kept under agitation for 30 minutes, and then filtered, washed with ultrapure water and inserted into 50 mL of 0.02% (v/v) glutaraldehyde solution at 4 °C for 30 minutes, for crosslinking. Finally, the granules

Formulation	Sodium alginate (% m/v)	Starch (% m/v)	Pectina (% m/v)	Glutaraldehyde (% v/v)	HRP(U/mL)
White	4	-	-	0,02	-
Alginate	4	-	-	0,02	7,5
Alginate-pectin	4	-	1	0,02	7,5
Alginate-pectin-starch	4	1	1	0,02	7,5

Table 1 - Composition of different alginate gel formulations for enzyme immobilization

Formulation	Initial			7 days		
	Production (%)	Efficiency (%)	Activity recovered (%)	Production (%)	Efficiency (%)	Activity recovered (%)
Alginate	61,31 ± 0,64^{bA}	15,86 ± 0,17^{bB}	9,72 ± 0,01^{bB}	61,01 ± 0,16^{bA}	19,04 ± 0,05^{aA}	11,62 ± 0,01^{aA}
Alginate-pectin	82,26 ± 1,74^{aA}	6,83 ± 0,14^{cB}	5,62 ± 0,01^{cB}	70,39 ± 2,18^{aB}	14,77 ± 0,45^{cA}	10,39 ± 0,01^{cA}
Alginate-pectin-starch	61,65 ± 1,15^{bA}	18,18 ± 0,34^{aA}	11,21 ± 0,01^{aA}	61,33 ± 0,66^{bA}	17,14 ± 0,19^{bB}	10,51 ± 0,01^{bB}

Mean ± standard deviation. Different lowercase letters in the same column indicate a significant statistical difference ($p<0.05$) for the same parameter between the different formulations. Different capital letters on the same line for the same parameter indicate a significant statistical difference ($p<0.05$) between the times evaluated (initial and 7 days) for the same formulation.

Table 1 - Yield, efficiency and enzymatic activity recovered after the HRP immobilization process in alginate, alginate-pectin and alginate-pectin-starch (initial time - 0 h and 7 days).

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were washed extensively with ultrapure water until no proteins were present in the washing solutions (by reading the absorbance on a UV-Vis spectrophotometer at 280 nm), and then stored in polypropylene tubes at 4 °C and protected from light.

PERFORMANCE, IMMOBILIZATION EFFICIENCY AND ACTIVITY RECOVERY

The yield, immobilization efficiency and recovery of enzymatic activity were evaluated according to the methodology proposed by Sheldon and Van Pelt (2013). All these parameters were evaluated at the initial time (right after preparation of the immobilized HRP granules) and after 7 days of storage. The yield was calculated through the relationship between the immobilized activity (total initial activity available for immobilization subtracted from the total enzymatic activity of the remaining immobilization solutions) and the total initial activity of the free enzyme solution, according to Equation 2.

$$Production\ (\%) = \frac{Immobilized\ activity}{Initial\ activity} * 100$$

(Equation 2)

To determine the immobilization efficiency, the relationship between the activity observed on the support (corresponding to the enzymatic activity that remained after immobilization) and the immobilized activity was used, according to Equation 3.

$$Efficiency\ (\%) = \frac{Observed\ activity}{Immobilized\ activity} * 100$$

(Equation 3)

The recovery of activity corresponds to the immobilization yield multiplied by the immobilization efficiency. This parameter allows one to have a general idea of the success of the immobilization, being calculated according to Equation 4.

$$Activity\ recovery\ (\%) = \frac{Production\ (\%) * Efficiency\ (\%)}{100}$$

(Equation 4)

STATISTICAL ANALYSIS

All results obtained were subjected to analysis of variance (ANOVA) and Tukey's test at 95% confidence, using the Paleontological Statistics Software Package for Education and Data Analysis - PAST 4.3 (HAMMER; HARPER; RYAN, 2001).

RESULTS AND DISCUSSION

In this work, HRP was immobilized on an alginate-pectin and alginate-pectin-starch hybrid support. For comparison purposes, enzymatic immobilization was also carried out on granules without the presence of starch and/or pectin. The parameters yield, efficiency and activity recovered for the immobilization process were evaluated at the initial time (0 h) and after 7 days, seeking to evaluate the stability of the derivative. The results obtained are shown in Table 1.

From the results obtained for time 0 h, it was observed that the formulation that presented the highest immobilization yield was the one containing alginate-pectin (82.26%), indicating that the combination of these two biopolymers contributed to greater fixation/ immobilization of the HRP on the support. Although the alginate-pectin formulation presented the highest yield, this formulation, in the present study, resulted in the lowest immobilization efficiency, that is, despite there being a considerable percentage of enzyme immobilized, only a small amount of enzyme remained functional. The recovery of activity was greatly influenced by low efficiency, resulting in 5.62%. Matto, Satar and Husain (2009) immobilized peroxidase from bitter gourd (Momordica charantia) and concavalin A in alginate-pectin granules (2.5% alginate and 2.5% pectin) and observed

an efficiency of 51%, a value higher than that observed in the present study (6.83%). The greater efficiency observed by Matto, Satar and Husain (2008) may be related to differences in the composition of the alginate gel or the characteristics of the immobilized enzyme.

The granules immobilized in alginate-pectin-starch showed an immobilization yield of 61.65%, efficiency of 18.18% (higher than the alginate-pectin composition) and activity recovery of 11.21%. Thus, when all parameters were observed together (yield, efficiency and activity recovery) for time 0 h, the best composition was alginate-pectin-starch, being superior to the control (alginate). These results indicate that the addition of starch to the composition contributed to the immobilized HRP remaining with greater catalytic activity, or even so that the granules formed presented lower resistance to substrate/product diffusion, greater mass transfer and affinity with the substrate. (IMAM; MARR C. P; MARR C. A., 2021).

After seven days of storage, HRP granules immobilized in alginate-pectin showed a reduction in immobilization yield compared to time 0 h, while for alginate-pectin-starch and control (alginate) similar values were observed (no statistically significant difference). The observed reduction can be justified by the enzymatic leakage of HRP from the support matrix to the external environment, a problem commonly reported and observed in immobilizations of this enzyme (MATTO; HUSAIN, 2009; URREA et al., 2021). Regarding efficiency, an increase can be seen after 7 days in relation to time 0 h for the alginate-pectin formulation and the control, related to internal structural changes in the derivative granules, in order to enable greater catalytic activity. Furthermore, the alginate-pectin-starch granules showed a reduction of around 1% in catalytic activity after 7 days, which can be justified by structural changes in the alginate granules or enzymatic denaturation. A reduction in catalytic activity over storage time is commonly observed in enzyme immobilizations, however, less pronounced than for free enzymes (BILAL; ASGHER, 2015; YAPAOZ; ATTAR, 2020; LATIF et al., 2022).

Reflecting the results observed for yield and efficiency, an increase in recovered enzyme activity was observed for alginate-pectin and control granules, and a reduction for alginate-pectin-starch, so that after 7 days the control formulation had presented the highest activity recovery value (11.62%), followed by alginate-pectin-starch granules (10.51%) and alginate-pectin (10.39%). In general, the addition of pectin and starch to alginate gels has been shown to be a potential alternative for enzyme immobilization. However, it is important to highlight that the present study consists of an initial investigation, so that further research using starch and pectin in the composition of hybrid biopolymers is necessary, as well as the evaluation of other parameters (such as optimal pH and temperature, kinetics and thermodynamics, storage time and potential applications) and/or other enzymes for immobilization, so that the employability of these materials in enzymatic immobilizations can be verified with greater basis.

CONCLUSION

From the tests carried out in the present study, it can be concluded that the addition of pectin to alginate, forming an alginate-pectin hybrid gel, contributes to a greater immobilization yield. However, problems such as low efficiency and enzyme leakage are observed. When starch is added to the composition, in addition to pectin (alginate-pectin-starch), there is a lower immobilization yield than that observed for alginate-pectin, however, a higher immobilization efficiency. After seven days of storage, the alginate-

pectin-starch granules did not show significant enzymatic leakage, while the alginate-pectin granules showed around 11.9% leakage. On the other hand, enzymatic efficiency was higher for alginate-pectin granules after seven days compared to time 0 h, and lower for alginate-pectin-starch, indicating that structural and conformational changes occur during storage time, influencing catalytic activity. Finally, it is highlighted that more in-depth studies, as well as the evaluation of other immobilization parameters and fixed assets, are necessary.

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