The Immobilization and Stabilization of Trypsin from the Porcine Pancreas on Chitosan and Its Catalytic Performance in Protein Hydrolysis

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Abstract: In this study, trypsin from the porcine pancreas was immobilized on a heterofunctional support prepared by activating chitosan (Chit) hydrogel with glutaraldehyde (GA), then functionalizing it with glycine (Chit–GA–Gly). The catalytic performance of the immobilized trypsin in the hydrolysis reactions was compared with the catalytic performance of the immobilized enzyme on glutaraldehyde-activated chitosan (Chit–GA) and chitosan hydrogel (Chit). The maximum concentration of immobilized protein on Chit–GA–Gly was approximately 16 mg·g\(^{-1}\) at pH 9.0 (5 mmol·L\(^{-1}\) buffer sodium carbonate) at 25 °C from an offered protein loading of 20 mg·g\(^{-1}\). This biocatalyst exhibited maximum specific activity (SA) of 33.1 ± 0.2 nmol·min\(^{-1}\)·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·min\(^{-1}\)·mg\(^{-1}\) and 8.1 ± 0.1 nmol·min\(^{-1}\)·mg\(^{-1}\)). The Elovich kinetic model was used to describe the adsorption process using low (3 mg·g\(^{-1}\)) and high (20 mg·g\(^{-1}\)) initial protein loadings. The optimal temperature for BAPNA hydrolysis catalyzed by the immobilized trypsin (60 °C) was 10 °C higher than that of its soluble form. Additionally, the immobilized enzyme was 16 to 20 times more stable than its soluble form at 50–55 °C. Thermodynamic studies were conducted to elucidate the kinetics of the thermal inactivation process of soluble and immobilized forms. Complete hydrolysis of bovine serum albumin (BSA) at 37 °C was achieved after 2 h using a soluble enzyme, while for its immobilized form, the hydrolysis yield was 47%. Reuse tests revealed that this biocatalyst retained 37% of its original activity after 10 successive hydrolysis batches. Based on these results, this support could be used as an interesting alternative for producing heterogeneous biocatalysts with high catalytic activity and thermal stability when producing protein hydrolysates.

Keywords: trypsin from porcine pancreas; immobilization; heterofunctional support; thermodynamics studies; protein hydrolysis

1. Introduction

Trypsin (EC 3.4.21.4) belongs to the serine endoprotease family 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 ofzymogens, namely chymotrypsinogen and procarboxypeptidase [1]. When compared to other trypsins, e.g., bovine or human trypsin, trypsin from the porcine pancreas exhibits activity in protein hydrolysis [2]. Its structure consists of a long peptide chain of 223 amino acids, featuring a catalytic site with the typical triad of serine (Ser195), histidine (His57), and aspartate (Asp102), as shown in
Figure 1. Specifically, it hydrolyzes the carboxy-terminal (C-terminal) bonds of cationic amino acids, arginine, and lysine [3]. Given its digestive enzyme nature and high specificity, trypsin has numerous biochemical and industrial applications, allowing for better control when hydrolyzing proteins [2,4,5]. This enzyme has been utilized in leather processing and proteome analysis to reduce food protein allergies, generate bioactive peptides, and prepare easily digestible dairy products for infants [6–10]. However, despite its various industrial applications, certain limitations do exist, e.g., 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 [11–13]. To address these limitations, various enzyme immobilization strategies have been proposed that would allow for the repeated use of enzymes like trypsin from porcine pancreas, thereby facilitating efficient recovery from reaction mediums, simplifying separation processes, and reducing contamination risks in both the enzymes and final products [14–21]. Immobilized enzymes, compared to their soluble counterparts, may show enhanced stability if properly performed [17,22], increasing the operation window. Notably, trypsin immobilization inside porous supports drastically minimizes the autolysis process in proteases like trypsin [23–25].

Chitosan has been extensively utilized as a support matrix for enzyme immobilization [27–31]. This biomaterial is an abundant by-product of the fishing industry [32] and has specific applications in controlled-release drugs, functional foods, and food preservatives [33]. Chitosan is highly suitable for enzyme immobilization given that it is non-toxic, easily biodegradable, and highly biocompatible and has a strong affinity for proteins [34]. Its structure contains different groups like hydroxyl and amino, which further facilitate enzyme immobilization [35,36]. Nevertheless, modifying its chemical structure is preferred when trying to obtain a more chemically resistant material. Additionally, if it covalently immobilizes the enzyme on the support, its amino groups are modified with diverse groups able to covalently react with enzymes, becoming a heterofunctional support [16,37].
Glutaraldehyde is one of the most commonly used bifunctional agents for activating aminated supports like chitosan in enzyme immobilization processes [38]. Through covalent bonding, it interacts with various chemical groups present in the protein structure, particularly primary amino groups, thiols, phenols, and imidazole [38–42]. The terminal amino group of a protein used to exhibit a lower pKₐ than the ε-amino group of Lys (pKₐ ≈ 6.5–8.5 depending on the vicinal amino acid) is typically the most reactive amino group in a protein, facilitating its covalent attachment to the support via the glutaraldehyde chemistry at neutral pH values.

In this study, the objective was to design a heterofunctional support derived from chitosan gel (Chit–GA–Gly) for immobilizing trypsin from the porcine pancreas. This support will present cationic groups from the support and the Gly, anionic groups from the Gly, and some hydrophobicity from glutaraldehyde moieties, providing that way a string adsorption. The physically immobilized trypsin will be subsequently used to hydrolyze BSA in a batch system [43]. This heterofunctional support was prepared via sequential activation of chitosan hydrogels with glutaraldehyde, followed by functionalization of the glutaraldehyde with glycine, and it was first utilized to efficiently immobilize a microbial lipase, displaying high catalytic activity and operational stability in an esterification reaction [30]. Importantly, our literature review in the Scopus database did not reveal any previous applications of this support for the immobilization of proteases, including trypsin from porcine pancreas, highlighting the novel contribution of this study. The investigation entails evaluating the influence of pH on protein immobilization and loading and conducting kinetic studies of adsorption on the prepared support. Furthermore, this study includes an assessment of the influence of temperature on enzyme activity and its thermal stability and determines the kinetic and thermodynamic parameters. Both soluble and immobilized trypsin preparations were employed for the hydrolysis of the BSA protein, which was selected due to its water solubility and well-known structure [44]. To assess the practical applicability of this biocatalyst, reuse tests were conducted in a sequential batch system. Unlike typical assays that use small synthetic substrates [45], this study employed a substrate that closely resembles a real-case scenario for a (BSA) substrate used in industry to obtain peptides [46].

2. Results and Discussion

2.1. Influence of the Solution pH on Trypsin Immobilization on the Different Supports

In this study, the influence of solution pH (ranging from 4.0 to 10.0) on the enzyme immobilization process was initially evaluated using Chit–GA–Gly. The isoelectric point (pI) of trypsin from the porcine pancreas ranges from 10.1 to 10.5 [47], while the support Chit–GA–Gly pI is pH 4.7 [30]. Its performance was then compared with that of chitosan hydrogel without additional activation steps, and the classic support chitosan hydrogel was activated with glutaraldehyde (Chit–GA). These tests were conducted at low ionic strength (5 mmol·L⁻¹) to avoid potential competition between the ions in the solution and the ionic groups present on the surface and protein of the three tested supports, all under high protein loading (20 mg·g⁻¹). The immobilization parameters for the different prepared heterogeneous biocatalysts are summarized in Table 1.

Based on the results, the adsorption capacity of the newly prepared support (Chit–GA–Gly) increased with the solution pH. This fits with the high isoelectric point of trypsin (between 10.1 and 10.5) [47]; the protein will mainly have a cationic character at a pH lower than 10. Under acidic conditions (pH between 4.0 and 6.0), we observed the lowest amount of immobilized protein. In a neutral pH medium, an increase in the amount of immobilized protein may be observed (See Table 1). 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 group of Gly, but remark-
At pH 9.0, the medium in which the support exhibited the highest adsorption capacity (SA = 33.1 ± 0.2 nmol min⁻¹·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 through ionic interaction is one of the mechanisms involved in the immobilization process [30]. 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 glycine. Immobilization at pH higher than the support pI gives the support a negative charge (high density of negative charges from the deprotonation of the carboxylate groups by functionalization with glycine). Consequently, the interaction between the enzyme and the support is influenced by preferential interactions between these groups and the protonated amino groups on the enzyme surface. At pH 9.0, the medium in which the support exhibited the highest adsorption capacity and catalytic performance (expressed in HA and SA values), the interaction between enzyme and support may involve the 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 glycine, 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 [48].

Table 1. Influence of pH on the immobilization parameters of trypsin from porcine pancreas on chitosan (Chit), chitosan–glutaraldehyde (Chit–GA), and chitosan–glutaraldehyde–glycine (Chit–GA–Gly) hydrogels.

<table>
<thead>
<tr>
<th>Support</th>
<th>pH</th>
<th>IP ⁴ (mg g⁻¹)</th>
<th>IY ⁵ (%)</th>
<th>HA ⁶ (nmol min⁻¹·g⁻¹)</th>
<th>SA ⁷ (nmol min⁻¹·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chit–GA–Gly</td>
<td>4.0</td>
<td>6.8 ± 0.2</td>
<td>30.7 ± 0.5</td>
<td>18.1 ± 2.0</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.4 ± 0.3</td>
<td>25.6 ± 1.1</td>
<td>29.7 ± 0.8</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>6.8 ± 0.1</td>
<td>32.4 ± 0.5</td>
<td>45.5 ± 0.9</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>9.1 ± 0.3</td>
<td>44.5 ± 1.1</td>
<td>115.5 ± 3.7</td>
<td>17.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>11.7 ± 0.1</td>
<td>56.2 ± 0.2</td>
<td>163.3 ± 2.4</td>
<td>23.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>16.2 ± 0.2</td>
<td>81.0 ± 0.2</td>
<td>224.8 ± 1.6</td>
<td>33.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>15.1 ± 0.1</td>
<td>71.6 ± 0.2</td>
<td>117.2 ± 5.9</td>
<td>26.1 ± 0.9</td>
</tr>
<tr>
<td>Chit</td>
<td>8.0</td>
<td>2.1 ± 0.4</td>
<td>10.5 ± 1.8</td>
<td>57.6 ± 5.7</td>
<td>26.9 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>3.8 ± 0.3</td>
<td>19.8 ± 1.4</td>
<td>84.3 ± 2.6</td>
<td>21.9 ± 0.7</td>
</tr>
<tr>
<td>Chit–GA</td>
<td>7.0</td>
<td>20.0</td>
<td>100.0</td>
<td>161.5 ± 1.1</td>
<td>8.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>20.0</td>
<td>100.0</td>
<td>139.3 ± 2.2</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>19.1 ± 0.2</td>
<td>98.6 ± 0.8</td>
<td>129.1 ± 2.1</td>
<td>6.7 ± 0.1</td>
</tr>
</tbody>
</table>

⁴—Immobilized protein concentration; ⁵—Immobilized yield; ⁶—Hydrolytic activity; ⁷—Specific activity.

Moreover, hydrophobic interactions generated by the presence of glutaraldehyde molecules also seem to play a critical role in the immobilization process [30]. 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 (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 compared to the hydrolytic activity values obtained when using Chit–GA–Gly. By contrast, the specific activity values 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.

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 the first ion exchange/hydrophobic
interactions, followed by the formation of covalent bonds between enzyme reactive groups and the glutaraldehyde groups introduced into the support [30]. Under these experimental conditions, we observed maximum hydrolytic activity at pH 7.0. Regarding specific activity, these values were almost two times lower than those obtained for the Chit–GA–Gly heterofunctional support. Consequently, the enzyme immobilization pH value was fixed at pH 9.0 using Chit–GA–Gly as the support. The Chit and Chit–GA supports were excluded from subsequent studies due to the superior catalytic activity values of the biocatalyst prepared using Chit–GA–Gly.

2.2. Influence of Time on the Immobilization Process: Kinetic Adsorption Studies

In this study, the influence of immobilization time on the enzyme immobilization process on Chit–GA–Gly was evaluated using low (3 mg·g⁻¹) and high (20 mg·g⁻¹) initial protein loadings. These tests were conducted in 5 mmol·L⁻¹ sodium carbonate at pH 9.0 and 25 °C, with mechanical stirring at 200 rpm. As shown in Figure 2A–C, increasing the contact time increases the amount of enzyme adsorbed in the support.

Equilibrium in the adsorption process conducted at low protein loading (3 mg·g⁻¹) was reached after 60 min of contact. However, the immobilization process, when performed with a high protein loading (20 mg·g⁻¹), only reached equilibrium after 300 min of contact time due to possible enzyme molecule aggregation and increased viscosity in the reaction medium, thus requiring longer contact times. To gain a better understanding of the enzyme adsorption process on the prepared heterofunctional support, the non-linear kinetic pseudo-first-order, pseudo-second-order, and Elovich models were fitted to the resulting experimental data. The correlation coefficient (R²) was used to select the most appropriate kinetic model for the adsorption process. As shown, the kinetic pseudo-first-order (Figure 2A) and pseudo-second-order (Figure 2B) models produced the lowest R² values for the two loadings evaluated (0.7863 ≥ R² ≥ 0.9483). However, the Elovich kinetic model (Figure 2C) produced the highest correlation coefficient values (R² = 0.9748 for 3 mg·g⁻¹ loading and R² = 0.9532 for 20 mg·g⁻¹ loading) and was thus selected to describe the kinetics of trypsin immobilization on the selected support. This model does not predict any defined mechanism for the adsorption process due to the high heterogeneity of the support [49], resulting from the introduction of different reactive groups onto the surface, as reported above. Enzyme immobilization through various interactions can contribute to stabilization, which is an essential requirement when preparing highly stable and active biocatalysts.

![Figure 2](image-url)
2.3. Influence of Temperature on Soluble and Immobilized Trypsin Activity

The influence of temperature on the activity of both soluble and immobilized trypsin was investigated between 25 and 80 °C. This study was performed in 5 mmol·L\(^{-1}\) sodium phosphate at pH 8.0, with continuous mechanical stirring at 200 rpm (Figure 3).

![Figure 3. Influence of temperature on the activity of soluble and immobilized trypsin from porcine pancreas on Chit–GA–Gly with an initial protein loading of 3 mg·g\(^{-1}\) at pH 8.0 (50 mmol·L\(^{-1}\) buffer sodium phosphate) using BAPNA as substrate, as described in the methodology. Relative activity of 100% corresponds to 86.6 nmol·min\(^{-1}\)·mL\(^{-1}\) for the soluble enzyme and 263.4 nmol·min\(^{-1}\)·g\(^{-1}\) for the immobilized trypsin.](image)

The maximum relative activity for soluble trypsin was observed at 50 °C, while for immobilized trypsin, it was found at 60 °C. Within the temperature range of 55–80 °C, the immobilized trypsin displayed higher relative activity than the soluble enzyme. These results provide clear evidence of the enhanced thermal stabilization of the enzyme following immobilization on the heterofunctional support, as described earlier, through various interactions.

2.4. Thermal Stability Tests and Estimating Kinetic and Thermodynamic Parameters

Thermal stability tests were conducted on trypsin from the porcine pancreas in both its soluble and immobilized forms. The investigation took place at pH 7.0 (50 mmol·L\(^{-1}\) sodium phosphate) and covered a temperature range from 37 to 55 °C. As depicted in Figure 4A–D, enzyme inactivation increased with incubation temperature.

The thermal inactivation constants (kd) showed a gradual increase, accompanied by a decrease in half-life values (t\(_{1/2}\)) as the temperature increased (Table 2). For soluble trypsin, k\(_d\) values ranged from 0.01262 min\(^{-1}\) (t\(_{1/2}\) = 340 min) at 37 °C to 0.14721 min\(^{-1}\) (t\(_{1/2}\) = 4.5 min) at 55 °C, while for immobilized trypsin, the range was 0.00077 min\(^{-1}\) (t\(_{1/2}\) = 2100 min) at 37 °C to 0.01600 min\(^{-1}\) (t\(_{1/2}\) = 72 min) at 55 °C.
were performed at pH 7.0 (50 mmol·L\(^{-1}\) buffer sodium phosphate). Relative activity of 100% (or \(a_r = 1\)—see Equation (7)) corresponds to initial hydrolytic activity for soluble (86.6 nmol·min\(^{-1}\)·mL\(^{-1}\)) and immobilized (263.4 nmol·min\(^{-1}\)·g\(^{-1}\)) trypsin.

**Figure 4.** Thermal inactivation curves of soluble (■) and immobilized (□) trypsin from porcine pancreas per incubation at 37 °C (A), 45 °C (B), 50 °C (C), and 55 °C (D). Inactivation tests were performed at pH 7.0 (50 mmol·L\(^{-1}\) buffer sodium phosphate). Relative activity of 100% (or \(a_r = 1\)—see Equation (7)) corresponds to initial hydrolytic activity for soluble (86.6 nmol·min\(^{-1}\)·mL\(^{-1}\)) and immobilized (263.4 nmol·min\(^{-1}\)·g\(^{-1}\)) trypsin.

**Table 2.** Estimation of apparent kinetic and thermodynamic parameters of thermal inactivation of soluble and immobilized trypsin from porcine pancreas at different temperatures.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Biocatalyst</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R^2)</td>
<td>S(^a)</td>
<td>0.97521</td>
</tr>
<tr>
<td></td>
<td>I(^b)</td>
<td>0.96722</td>
</tr>
<tr>
<td>(k_f) (min(^{-1}))</td>
<td>S(^a)</td>
<td>0.01262</td>
</tr>
<tr>
<td></td>
<td>I(^b)</td>
<td>0.00077</td>
</tr>
<tr>
<td>(t_{1/2}) (min)</td>
<td>S(^a)</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>I(^b)</td>
<td>2100</td>
</tr>
<tr>
<td>(SF)</td>
<td>S(^a)</td>
<td>6.2</td>
</tr>
<tr>
<td>(E_d) (kJ·mol(^{-1}))</td>
<td>S(^a)</td>
<td>121.7</td>
</tr>
<tr>
<td>(\Delta H) (kJ·mol(^{-1}))</td>
<td>I(^b)</td>
<td>119.1</td>
</tr>
<tr>
<td>(\Delta G) (kJ·mol(^{-1}))</td>
<td>S(^a)</td>
<td>144.6</td>
</tr>
<tr>
<td>(\Delta S) (kJ·mol(^{-1}))</td>
<td>I(^b)</td>
<td>85.9</td>
</tr>
<tr>
<td>(\Delta S) (J·mol(^{-1})·K(^{-1}))</td>
<td>S(^a)</td>
<td>93.1</td>
</tr>
<tr>
<td>(\Delta S) (J·mol(^{-1})·K(^{-1}))</td>
<td>I(^b)</td>
<td>106.9</td>
</tr>
<tr>
<td>(\Delta S) (J·mol(^{-1})·K(^{-1}))</td>
<td>I(^b)</td>
<td>166.0</td>
</tr>
</tbody>
</table>

\(^a\)—Soluble trypsin; \(^b\)—Immobilized Trypsin.
The apparent thermodynamic parameters of thermal inactivation were also determined and are summarized in Table 2. At 50 °C, around 40% of the initial activity was retained by soluble trypsin after 12 min of incubation, while immobilized trypsin showed similar activity after 3 h of incubation. The maximum stabilization factor, approximately 20 times, was observed at 40 °C and 50 °C, followed by incubation at 55 °C (SF = 16) and 37 °C (SF = 6.2). The estimation of thermodynamic parameters provides essential information about the mechanism of enzyme inactivation [50]. The thermal inactivation energy (\(E_d\)) values for soluble and immobilized trypsin were determined by plotting the natural logarithm values of the thermal inactivation constant (\(\ln k_d\)) versus the inverse of the absolute temperature (1/\(T\))—Arrhenius Equation (Figure 5). The thermal inactivation energy values for soluble and immobilized trypsin were determined to be 121.7 and 147.2 kJ mol\(^{-1}\), respectively. These results demonstrate that immobilized trypsin requires higher inactivation energy compared to the soluble enzyme. These findings are consistent with previous studies reported in the literature [51–55].

Table 2 shows the values of the apparent thermodynamic parameters, including enthalpy (\(\Delta H^\#\)), Gibbs energy (\(\Delta G^\#\)), and entropy (\(\Delta S^\#\)). The soluble and immobilized forms of trypsin exhibited \(\Delta H^\#\) values of approximately 119 and 144 kJ mol\(^{-1}\), respectively. These results indicate that more energy is required for the thermal inactivation of the enzyme in its immobilized form [56,57], as previously mentioned. Moreover, the positive values indicate that the thermal inactivation process is endothermic, requiring thermal energy to break intermolecular interactions, such as hydrogen bonds, hydrophobic, and/or ionic interactions, that stabilize the native enzyme structure [58,59].

The immobilized enzyme also showed higher \(\Delta G^\#\) values compared to the soluble enzyme, suggesting an increased resistance of the immobilized enzyme towards thermal distortion within the evaluated temperature range (37 °C to 55 °C). As presented in Table 2, the positive values obtained for \(\Delta S^\#\) ranged from 106.9 to 111.5 J mol\(^{-1}\) K\(^{-1}\) and 166.0 to 174.2 J mol\(^{-1}\) K\(^{-1}\) for soluble and immobilized trypsin, respectively. These positive \(\Delta S^\#\) values confirm an irreversible chemical modification in the polypeptide chains of the enzymes, resulting in random structures and increased disorder in their configurations [58,60]. This finding also indicates that the prepared biocatalyst was thermodynamically more stable than the soluble form of the enzyme, confirming the superior thermal stabilization of immobilized trypsin compared to its soluble form.
2.5. Evaluating the Catalytic Performance of Soluble and Immobilized Trypsin in the BSA Hydrolysis Reaction and Operations Stability Tests

In this study, the catalytic performance of soluble and immobilized trypsin in the hydrolysis of BSA was analyzed. Previously, tests were performed with the soluble enzyme to measure the time required for maximum conversion of the hydrolysis product. The determined reaction time was 2 h. Therefore, the soluble and immobilized enzyme experiments were conducted for 2 h, as depicted in Figure 6.

![Figure 6. Hydrolysis profile of BSA catalyzed by soluble trypsin from porcine pancreas immobilized at 37 °C in the absence of buffer (distilled water—pH 8.0).](image)

Figure 6 illustrates the BSA hydrolysis profiles for both soluble and immobilized trypsin after 120 min, where the maximum conversion reached 100% and 47%, respectively, representing the release of 381.3 nmol and 178.1 nmol of α-amino groups per mg of protein. Using the immobilized enzyme, an apparent lag time is clearly visible—a lag time that is not visualized using the free enzyme. In the case of protein hydrolysis, there are some problems. Firstly, the substrate is very large; in this way, only properly oriented enzyme molecules can act against it, while all enzyme molecules can attack the small synthetic substrate. Once the proteases with proper orientation start to hydrolyze BSA, the fragments of smaller size can be attacked by other immobilized enzyme molecules [61–63]. Moreover, the large size of BSA will increase the lack of substrate for the inner immobilized enzyme molecules caused by substrate diffusional problems; also, the reduction of the BSA size along the reaction can reduce this matter [61–63]. However, there is no clear exponential reaction course. These results demonstrate that performing hydrolysis using the new immobilized enzymes is feasible, although it is slower. Despite potential issues like diffusion difficulties and subsequent reduction in reaction speed, it is important to consider the advantages of handling the heterogeneous biocatalyst with ease and the possibility of its reuse.

The reuse of immobilized enzymes is a crucial aspect of their applicability in industrial processes. To demonstrate the benefits of the immobilization procedure, we conducted a study on the reuse of the biocatalyst by performing successive BSA hydrolysis batches, as previously described. As shown in Figure 7, the relative activity of trypsin immobilized on modified chitosan (Chit–GA–Gly) gradually decreased with an increasing number of batches. During the first three batches, the biocatalyst retained 97% of the hydrolysis percentage in the first cycle. However, from the fourth to the sixth batch, there was a decline in the hydrolysis percentage to approximately 69%, followed by a further decrease in the seventh and eighth batches to approximately 55%. Finally, after the tenth batch, the immobilized trypsin retained 35% of its initial activity. One possible explanation for the gradual loss of catalytic activity is the accumulation of substrate and/or unconverted
products adsorbed on the enzyme or the support that can partially block the access of the DSA molecules to the active center of the enzyme. Similar results have been observed in previous studies documented in the specialized literature [64–67].

Figure 7. Operational stability (reuse) tests of trypsin from porcine pancreas immobilized on Chit–GA–Gly in the hydrolysis of BSA after successive batches of 120 min each. The hydrolysis reactions were conducted at 37 °C in the absence of buffer agents.

3. Materials and Methods

3.1. Materials

Trypsin from porcine pancreas (T-0303, Type IX-S, lyophilized, 13,000–20,000 BAEE units·mg⁻¹ protein), benzoyl-DL-arginine-p-nitroanilide (BAPNA), p-nitroaniline (≥99%, Sigma Ref: 185310), o-phthalaldehyde (OPA), bovine serum albumin (BSA), and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were all 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—GA (25% v·v⁻¹); glycine (Gly); and sodium dodecyl sulfate (SDS) were purchased from Synth® (São Paulo, SP, Brazil). All other chemical reagents and organic solvents used in this study were of analytical grade and obtained from Synth®.

3.2. Preparing the Heterofunctional Supports

Glutaraldehyde-activated chitosan hydrogel (Chit–GA) was prepared based on a previous study [52]. A total of 5 g of powdered chitosan was added to 100 mL of a glacial acetic acid solution (5% v·v⁻¹) at 25 °C for 4 h under mechanical agitation at 1000 rpm. The resulting solution was then introduced via syringe into a 0.1 mol·L⁻¹ NaOH solution (Chit:NaOH 1:10 v·v⁻¹), subjected to mild mechanical stirring at 100 rpm, 25 °C, for 12 h to produce the chitosan hydrogel (Chit). Next, the suspension was filtered in a Buchner funnel, thoroughly rinsed with distilled water until pH 7.0, and stored in a 70% ethanol solution at 4 °C. Then, 10 g of chitosan hydrogel (Chit) was immersed in a glutaraldehyde solution prepared 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). The suspension was subjected to a continuous mechanical stirring (200 rpm) at 25 °C for 1 h to yield the glutaraldehyde-activated chitosan hydrogel (Chit–GA). This activated support was then filtered in a Buchner funnel with Whatman filter paper N° 41 and washed thoroughly with distilled water. Finally, 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 maintained under orbital agitation (200 rpm) for 48 h. The resulting support was filtered in
3.3. Immobilization of Trypsin on Chit–GA–Gly Hydrogel

The trypsin immobilization procedure on the supports was conducted following methods established in previous studies within our research group [50,68]. The immobilization process involved preparing a suspension by combining the enzymatic solution, previously mixed with a 5 mmol·L\(^{-1}\) buffer solution and the support in a 1:20 (m·v\(^{-1}\)) proportion (support/trypsin solution). The immobilization suspension was then incubated at 25 °C on an orbital shaker (200 rpm) with 12 h of contact time. The heterogeneous biocatalysts were prepared by offering initial protein loadings of 3 mg·g\(^{-1}\) of support (low loading) and 20 mg·g\(^{-1}\) (high loading). Once the enzyme was immobilized, the prepared biocatalysts were vacuum filtered using a Buchner funnel with Whatman filter paper N° 41 and thoroughly washed with distilled water. The adsorption process was monitored by measuring the decrease in protein concentration or hydrolytic activity in the supernatant. Subsequently, the prepared biocatalysts were stored at 4 °C in the refrigerator for 24 h.

The protein concentration was determined using the method described by Bradford [69], using BSA as a protein standard. The concentration of immobilized protein (IP—mg·g\(^{-1}\) of support) was calculated according to Equation (1) [68,70]:

\[
IP = \frac{V \times (C_0 - C_e)}{m} \tag{1}
\]

where \(V\) is the volume of the enzymatic solution (mL), \(C_0\) is the initial protein concentration (mg·mL\(^{-1}\)), \(C_e\) is the protein concentration after immobilization (mg·mL\(^{-1}\)), and \(m\) is the mass of the support (g).

The enzymatic activities of soluble and immobilized trypsin were measured by using BAPNA as substrate (919.7 \(\mu\)mol·L\(^{-1}\)) at pH 8.0 (50 mmol·L\(^{-1}\) sodium phosphate) at 37 °C under constant stirring (200 rpm) [71]. The reaction was carried out using 1.0 mL of BAPNA solution and 100 \(\mu\)L of enzyme solution in a quartz cuvette with a path length of 1 cm. The reaction mixture was incubated at 37 °C for 10 min and stopped by adding 250 \(\mu\)L of 30% glacial acetic acid (v·v\(^{-1}\)) into the medium. 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 specific activity (SA—nmol·min\(^{-1}\)·mg\(^{-1}\)) of the heterogeneous biocatalyst was determined by establishing the relationship between the enzymatic activity and the concentration of immobilized protein. (Equation (2)) [68,70]:

\[
SA = \frac{HA}{IP} \tag{2}
\]

where \(HA\) is the apparent hydrolytic activity of the prepared biocatalyst (nmol·min\(^{-1}\)·g\(^{-1}\) biocatalyst), and \(IP\) is the concentration of immobilized protein (mg enzyme·g\(^{-1}\) of biocatalyst).

The immobilization yield (IY) was determined according to Equation (3) [70]:

\[
IY = \frac{(HA_0 - HA_e) \times 100}{HA_0} \tag{3}
\]

where \(HA_0\) is the initial hydrolytic activity (nmol·min\(^{-1}\)·mL\(^{-1}\)), and \(HE_e\) is the residual hydrolytic activity in the supernatant after immobilization (nmol·min\(^{-1}\)·mL\(^{-1}\)).

3.4. Influence of pH on the Immobilization Process

The influence of pH on the immobilization parameters was investigated within the pH range of 4.0 to 10.0 (sodium acetate for pH 4.0 to 5.0, sodium phosphate for pH 6.0 to 8.0, and sodium carbonate for pH 9.0 to 10.0) at low ionic strength (5 mmol·L\(^{-1}\)). The experiments were conducted under agitation at 200 rpm, 25 °C, and 12 h of contact time.
3.5. Influence of Immobilization Time: Kinetic Adsorption Studies

The kinetics of trypsin from porcine pancreas adsorption on Chit–GA–Gly were studied under both low loading (3 mg·g⁻¹) and high loading (20 mg·g⁻¹) protein. The immobilization process was monitored by measuring the residual protein concentration in the supernatant of the immobilization suspension using the Bradford method [69]. Three conventional non-linear kinetic models, namely the pseudo-first-order (Equation (4)), pseudo-second-order (Equation (5)), and the Elovich models (Equation (6)) [49], were fitted to the experimentally obtained data:

\[ q_t = q_e \times \left(1 - e^{-k_1 \times t}\right) \quad (4) \]

\[ q_t = \frac{k_2 \times q_e^2 \times t}{1 \times k_2 \times q_e \times t} \quad (5) \]

\[ q_t = \frac{\ln (a + \beta)}{\beta} + \frac{\ln(t)}{\beta} \quad (6) \]

where \( q_t \) and \( q_e \) are the concentration of adsorbed trypsin at a given time \( t \) and at equilibrium (mg·g⁻¹), respectively; \( k_1 \) (min⁻¹) and \( k_2 \) (mg·g⁻¹·min⁻¹) are the first-order and second-order adsorption rate constants, respectively; \( a \) is the initial adsorption rate (mg·g⁻¹·min⁻¹); \( \beta \) is the desorption constant (g·g⁻¹·mg⁻¹·min⁻¹); and \( t \) is the contact time.

3.6. Influence of Temperature on the Activity of Soluble and Immobilized Trypsin

The influence of temperature on the activity of soluble and immobilized trypsin was evaluated using a loading of 3 mg·g⁻¹ to minimize diffusional effects on enzyme activity. The enzyme activity was assessed at various temperature values ranging from 25 °C to 80 °C, at increments of 5 °C. In this study, the highest observed hydrolytic activity value was taken as 100% (relative activity 100% corresponds to 86.6 nmol·min⁻¹·mL⁻¹ for the soluble enzyme and 263.4 nmol·min⁻¹·g⁻¹ for the immobilized trypsin). The reactions were conducted in 50 mmol·L⁻¹ sodium phosphate at pH 8.0.

3.7. Thermal Stability: Estimating the Kinetic and Thermodynamic Parameters

The thermal stability test for immobilized trypsin on Chit–GA–Gly with a loading of 3 mg·g⁻¹ to minimize diffusional effects on enzyme stabilization was initially conducted by incubating the immobilized enzyme in 50 mmol·L⁻¹ of sodium phosphate at pH 7.0 at temperatures ranging from 37 °C to 55 °C. Samples were periodically taken and immediately cooled in an ice bath to halt inactivation. Residual activity was assessed at 37 °C and pH 8.0, as described in Section 2.3. The initial hydrolytic activity was taken as relative activity 100% (=1 for this study, which corresponds to 86.6 nmol·min⁻¹·mL⁻¹ for the soluble enzyme and 263.4 nmol·min⁻¹·g⁻¹ for the immobilized trypsin). The inactivation constants \( (k_1) \) and half-lives \( (t_{1/2}) \) were determined using the non-linear exponential decay model proposed by Sadana and Henley—Equations (7) and (8), respectively [72]. The experimental data were analyzed using Origin Pro software, version 5.0.

\[ a_r = a + (1 - a) \times e^{-k_1 \times t} \quad (7) \]

\[ t_{1/2} = \frac{\ln(0.5 - a)}{k_1} \frac{(1 - a)}{a_r} \quad (8) \]

where \( a_r \) is the relative activity (dimensionless); \( a \) is the ratio between the specific activity of the final state (E₁) and the initial state (E); \( k_1 \) is the inactivation rate constant (min⁻¹); \( t \) is the thermal inactivation time (min); and \( t_{1/2} \) is the half-life (min).

The stabilization factor (SF) was calculated as the ratio between the half-lives of immobilized \( (t_{1/2}^{immobil}) \) and soluble \( (t_{1/2}^{soluble}) \) trypsin under the same experimental conditions, as shown in Equation (9):

\[ SF = \frac{t_{1/2}^{immobil}}{t_{1/2}^{soluble}} \quad (9) \]
The thermal inactivation energy ($E_d$) of soluble and immobilized trypsin was determined using the linearized Arrhenius equation (plot of $\ln k_d$ versus the inverse absolute temperature—1/$T$), as shown in Equation (10) [56]:

$$\ln k_d = \ln A - \frac{E_d}{R} \times \frac{1}{T} \times 10^3$$  \hspace{1cm} (10)

where $A$ is the frequency factor, and $R$ is the ideal gas constant ($8.314 \times 10^{-3}$ kJ·mol$^{-1}$·K$^{-1}$).

The enthalpy ($\Delta H^\#$), Gibbs energy ($\Delta G^\#$), and entropy ($\Delta S^\#$) values for the thermal inactivation of soluble and immobilized trypsin from the porcine pancreas were determined using the following equations (Equations (11)–(13)) [56]:

$$\Delta H^\# = E_d - RT$$  \hspace{1cm} (11)

$$\Delta G^\# = -RT \ln \left( \frac{k_d}{k_B} \times \frac{h}{T} \right)$$  \hspace{1cm} (12)

$$\Delta S^\# = \frac{\Delta H^\# - \Delta G^\#}{T}$$  \hspace{1cm} (13)

where $T$ is the absolute temperature (K), $h$ is the Planck constant ($6.626 \times 10^{-34}$ J·s or $1.104 \times 10^{-35}$ J·min), and $k_B$ is the Boltzmann constant ($1.3807 \times 10^{-23}$ J·K$^{-1}$).

3.8. BSA Hydrolysis Catalyzed by Soluble and Immobilized Trypsin and Operational Stability Tests

Hydrolysis experiments were performed by preparing BSA solutions under a fixed concentration (8 mg·mL$^{-1}$) in distilled water at pH 8.0. The proteolyses were performed in test tubes (10 mL) containing 1 mL of BSA solution immersed in a temperature-controlled water bath at 37 °C for 2 h under constant mechanical stirring (200 rpm), following the methodology described in a previous study [73]. Next, the biocatalysts were added to the reaction mixtures at a proportion of 0.32 mg protein, which corresponds to 20 mg of immobilized trypsin or 20 µL of soluble trypsin. Aliquots of the supernatant were collected at different time intervals. The extent of hydrolysis was determined by measuring the increase in free amino groups caused by the release of new peptides using the OPA reagent (o-phthaldialdehyde) [74]. Briefly, aliquots of samples ranging from 0 to 130 µL were mixed with 1.0 mL of freshly prepared OPA reagent. The OPA reagent was prepared daily by combining 25 mL of 100 mmol·L$^{-1}$ sodium tetraborate, 2.5 mL of 20% (m·v$^{-1}$) SDS, 40 mg of OPA in 1 mL of methanol, and 100 µL of $\alpha$-mercaptoethanol, and then adjusted to 50 mL with distilled water. After brief mixing, the absorbance at 340 nm was measured exactly after 2 min. A standard curve using L-Leucine was constructed to determine the number of primary amino groups related to the degree of hydrolysis (% H) of BSA, as shown in Equation (14) [73].

$$\% H = \frac{AAs - AAbe}{AAtm} \times 100$$  \hspace{1cm} (14)

where $AAs$ is the amount of free amino groups determined in the sample supernatant (mol); $AAbe$ is the amount of free amino groups determined in the enzyme blank supernatant—only enzymes and buffer as used in the sample assay (mol); and $AAtm$ is the total amount of $\alpha$-amino groups in the sample estimated from the total mass of protein in the sample and the average molecular mass of amino acids (113 g·mol$^{-1}$), where each amino acid represents an $\alpha$-amino group.

Biocatalyst reuse tests were also conducted, comprising ten successive BSA hydrolysis reaction cycles, each lasting 2 h, as previously reported in the BSA hydrolysis reaction. After each batch, the prepared heterogeneous biocatalyst was recovered through vacuum filtration on a Buchner funnel using Whatman filter paper N° 41. Subsequently, it was washed with distilled water to eliminate product and residual substrate molecules from the biocatalyst particles. The washed biocatalyst was then added to a fresh substrate solution to initiate a new reaction batch.
4. Conclusions

This study successfully utilized a chitosan-based heterofunctional support to immobilize an industrial enzyme, trypsin, from the porcine pancreas while retaining high catalytic activity, as indicated by the substantial concentration of immobilized protein. The tests also demonstrated that at a temperature (50 °C) where the biocatalyst exhibited maximum activity, the stabilization factor was 20 times higher compared to soluble trypsin. The different strategies employed for enzyme immobilization fostered strong interaction with the support, making it suitable for potential applications in industrial processes, such as protein hydrolysis, to obtain peptides. Notably, the immobilized trypsin exhibited excellent operational stability, retaining approximately 37% of its initial activity even after 10 successive batches of BSA hydrolysis. This effective immobilization process overcame inherent limitations associated with soluble enzyme molecules, such as solubility in water and limited reusability, thus providing enhanced structural stability and facilitating its use in industrial applications. Additionally, further studies can be conducted using the resulting protein hydrolysates, including bioactivity tests, to explore their antioxidant, antimicrobial, antihypertensive, and anti-inflammatory properties.


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References

8. Wang, X.; Chen, H.; Fu, X.; Li, S.; Wei, J. A Novel Antioxidant and ACE Inhibitory Peptide from Rice Bran Protein: Biochemical Characterization and Molecular Docking. *LWT* 2017, 75, 93–99. [CrossRef]


42. Kim, J.S.; Lee, S. Immobilization of Trypsin from Porcine Pancreas onto Chitosan Nonwoven by Covalent Bonding. *Polymers* 2019, 11, 1462. [CrossRef]


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