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RESEARCH ARTICLE

Immobilization of Serratiopeptidase on Polyaldehyde Dextran: Optimization of Immobilization Parameters and Enhancement of Controlled Proteolytic Performance

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Abstract

In this study, serratiopeptidase was covalently immobilized on polyaldehyde dextran (PAD) to improve handling stability and obtain a more reproducible proteolytic response under defined biochemical assay conditions. PAD was selected as a hydrophilic, water-soluble polysaccharide carrier because, unlike many particulate silica or inorganic supports, it permits mild aqueous Schiff-base coupling while providing a flexible hydrated microenvironment that may reduce enzyme self-association. PAD was prepared by periodate oxidation of dextran to an oxidation degree of approximately 25% - 30%, determined by hydroxylamine hydrochloride titration. Immobilization was performed at 20 - 25 °C for 6 - 24 h using enzyme-to-carrier ratios of 0.5 - 4.0 mg serratiopeptidase per 0.5 g PAD and coupling pH values of 4.0 - 10.0. Quantitative comparisons were performed as mean +/− SD from triplicate measurements and analyzed by one-way ANOVA with Tukey post hoc testing. The best functional balance was obtained at an enzyme-to-carrier ratio of 1:3 and pH 6.5 - 7.5, where apparent immobilization efficiency reached approximately 85% - 90% while proteolytic activity was retained. FTIR spectroscopy supported PAD formation and enzyme attachment through changes in aldehyde- and imine-associated bands, although these data are interpreted as supportive rather than conclusive structural proof. Casein digestion assays showed that PAD-immobilized serratiopeptidase produced a more proportional concentration-activity response and an apparent activity enhancement of 2.1 - 3.1-fold compared with the free enzyme. Apparent kinetic analysis gave Km values of approximately 0.52 mM for free serratiopeptidase and 0.84 mM for the immobilized preparation, with an apparent Vmax of about 0.96 umol/min for the immobilized form; kcat was not calculated because the catalytically accessible immobilized enzyme concentration was not directly determined. The immobilized preparation retained > 80% activity at pH 6.0 - 8.0, about 70% activity after 1 h at 50 °C, and about 85% activity after 30 days at 4 °C. These findings suggest that PAD is a useful polysaccharide carrier for stabilized serratiopeptidase systems, while future work should address direct leaching, reusability, substrate specificity, and application-specific validation.

Keywords: Bioconjugates, Enzyme immobilization, Polyaldehyde dextran, Polysaccharides, Proteolytic activity, Serratiopeptidase

Introduction

Proteolytic enzymes are important in pharmaceutical, biomedical, and industrial biotechnology because they catalyze selective cleavage of protein substrates and can be used for anti-inflammatory, fibrino(geno)lytic, mucolytic, antibiofilm, thrombolytic, and process-related applications [1-3,10,18,20]. Serratiopeptidase, a metalloprotease obtained from Serratia spp., is widely discussed as a multifunctional proteolytic enzyme with pharmaceutical relevance, particularly in formulations where anti-inflammatory, mucolytic, or fibrinolytic activity is desired [2,3]. However, practical use of the free enzyme is limited by environmental sensitivity, storage instability, and loss of predictable activity during handling. In solution, changes in pH, temperature, ionic strength, and enzyme concentration can promote conformational instability, aggregation, autoproteolysis, or self-interaction. These effects reduce not only total activity, but also dose-to-activity proportionality, which is critical for reproducible pharmaceutical formulation and industrial biocatalytic operation.

Immobilization is a widely used strategy for improving enzyme stability and process control, but its success depends on support chemistry, enzyme orientation, local hydration, substrate accessibility, and the degree of multipoint attachment [4,5,22]. Weak physical adsorption can cause enzyme leaching, whereas excessive covalent binding can restrict essential conformational motion or block access to macromolecular substrates. Therefore, for serratiopeptidase, the central biochemical challenge is to identify immobilization conditions that suppress aggregation and self-inhibition without creating excessive diffusional or steric limitations.

Common immobilization supports each have advantages and limitations. Chitosan and alginate matrices are biocompatible and useful for encapsulation or hydrogel formation, but their swelling, charge interactions, and release behavior can complicate direct interpretation of proteolytic activity [13-17]. Silica and other inorganic supports provide mechanical strength and defined surfaces, but they often require particle functionalization and careful safety or biocompatibility assessment. A recent TiO_2-serratiopeptidase bioconjugate provided an important enzyme-specific benchmark by improving stability and antibacterial performance [9]; however, that system was oriented toward nanoparticle functionality rather than a simple hydrophilic polysaccharide platform for proportional protease dosing. PAD was selected in the present study because it combines water solubility, mild aqueous chemistry, aldehyde-mediated covalent coupling, and a flexible dextran-derived microenvironment that may spatially separate enzyme molecules while maintaining substrate accessibility [6,7,21].

The working hypothesis was that immobilization on PAD could improve controlled proteolytic performance by 3 complementary effects: covalent anchoring through aldehyde-amino group coupling, spatial separation of enzyme molecules that reduces self-association, and a hydrated polysaccharide environment that improves residual activity under pH, thermal, and storage stress. At the same time, the study recognizes that imine linkages can be reversible and that immobilization may introduce mass-transfer limitations, especially when casein is used as a macromolecular substrate. For this reason, observed improvements are interpreted mainly as apparent functional stabilization and improved reproducibility rather than as definitive proof of intrinsic catalytic enhancement.

Accordingly, this study evaluates PAD-serratiopeptidase preparation in a structured way by examining: (i) PAD oxidation degree and FTIR-supported conjugate formation; (ii) enzyme-to-carrier ratio and coupling pH as immobilization variables; (iii) apparent immobilization efficiency and batch reproducibility; (iv) casein proteolytic activity and concentration-activity proportionality; (v) apparent kinetic parameters, including Km and Vmax where estimable; and (vi) residual activity after pH, thermal, and refrigerated storage challenges. In this manuscript, 'controlled proteolytic performance' is defined operationally as a more proportional concentration-activity response, lower assay variability, and greater residual activity after defined stress conditions.

Materials and methods

Materials

Serratiopeptidase (source: Serratia spp.; Roche Diagnostics GmbH, Germany) was used as the model

protease. Dextran (Mr approximately 40,000; Pharmacosmos, Denmark) was used for carrier synthesis. Sodium metaperiodate ($NaIO_4$, analytical grade), hydroxylamine hydrochloride, sodium hydroxide, sodium phosphate salts, sodium acetate, boric acid/borate buffer components, trichloroacetic acid, casein from bovine milk, Folin-Ciocalteu reagent, and tyrosine and tryptophan standards were obtained from commercial analytical-grade suppliers and used without further purification. Dialysis tubing with a molecular-weight cut-off of 12 - 14 kDa was used for removal of unbound enzyme and low-molecular-weight reagents. FTIR measurements were performed using a Bruker Vector-22 FTIR spectrometer with KBr pellet preparation. Catalog and batch numbers for locally supplied reagents are retained in the laboratory records; where supplier catalog numbers were not available from the regional distributor, the reagent grade and manufacturer information are reported to support reproducibility.

Synthesis of polyaldehyde dextran

Polyaldehyde dextran (PAD) was synthesized by periodate oxidation of dextran according to established oxidized-polysaccharide procedures with minor modifications [6,21]. Dextran was dissolved in acetate buffer (pH 4.25), and aqueous NaIO4 solution was added at a molar ratio dextran: IO_4^- = 1:1.5. The reaction was performed for 2 h at 25 °C in the dark to minimize periodate photodecomposition. The reaction mixture was transferred into 12 - 14 kDa dialysis tubing and dialyzed against distilled water for 24 h using at least 2 L of water per dialysis cycle, with water replacement every 4 h during the first 12 h and once after overnight incubation. The dialyzed product was lyophilized to obtain dry PAD. Aldehyde content was determined by hydroxylamine hydrochloride titration: A known mass of PAD was incubated with hydroxylamine hydrochloride solution, and the hydrochloric acid released during oxime formation was titrated with standardized NaOH. The degree of oxidation was calculated from NaOH consumption and expressed as mol aldehyde groups per mol glucose units. Under the selected conditions, the oxidation degree was approximately 25% - 30%, providing aldehyde functionality while preserving polymer solubility. The main possible cleavage routes are summarized in **Figure 1**. FTIR spectroscopy was used as a qualitative supporting method for aldehyde formation.

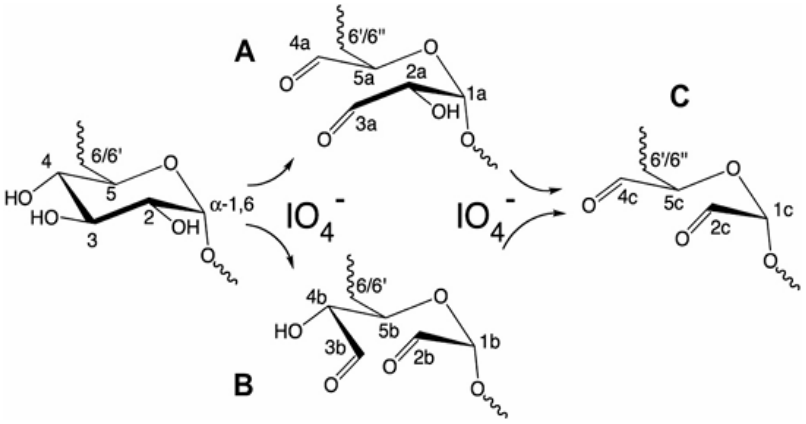


Figure 1 Possible periodate oxidation pathways of dextran leading to polyaldehyde dextran (PAD). Periodate cleaves vicinal diol groups in glucose residues to generate aldehyde functions that serve as reactive sites for later enzyme coupling. The scheme shows alternative cleavage routes, including C3-C4 and C2-C3 oxidation and double oxidation, which together explain how aldehyde-bearing dextran structures can form. Immobilization of serratiopeptidase.

For immobilization, 0.5 g of dry PAD was hydrated in 10 mL of 0.1 M buffer. Serratiopeptidase stock solution (5.0 mg/mL) was prepared in the same buffer and added to obtain enzyme-to-carrier ratios corresponding to 0.5, 1.0, 2.0, 3.0, and 4.0 mg enzyme per 0.5 g PAD. The coupling reaction was carried out at 20 - 25 °C for 6 - 24 h under gentle stirring. pH screening was performed at pH 4.0 - 10.0 using appropriate acetate, phosphate, and borate buffers to cover acidic, near-neutral, and mildly alkaline conditions while

avoiding strongly acidic media that may severely compromise serratiopeptidase structure. No reductive stabilization step with sodium borohydride or sodium cyanoborohydride was performed; therefore, the conjugate is described as a Schiff-base-linked PAD-serratiopeptidase preparation, and the possible reversibility of imine bonds is acknowledged as a limitation. After coupling, conjugates were dialyzed (12 - 14 kDa cut-off) against distilled water and washed until protein was not detectable in the washings by the Folin-Lowry assay, followed by lyophilization.

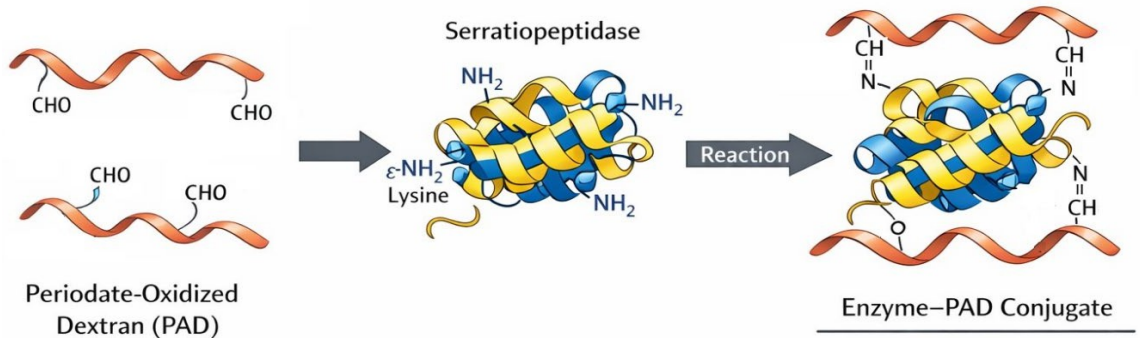


Figure 2 Proposed mechanism for serratiopeptidase immobilization on PAD through Schiff base formation. Aldehyde groups introduced into the dextran backbone react with accessible amino groups, mainly the epsilon-amino groups of lysine residues, on serratiopeptidase to generate imine (C=N) linkages. This covalent attachment anchors the enzyme within the hydrated polysaccharide matrix.

As illustrated in **Figure 2**, covalent immobilization is based on the reaction of periodate-oxidized dextran (PAD) with accessible amino groups on serratiopeptidase to form Schiff-base-linked enzyme-PAD conjugates.

Determination of immobilization efficiency

The apparent immobilization efficiency (%) was determined by measuring protein concentration in the initial enzyme solution, combined supernatant, and washing fractions before and after immobilization. Protein concentration was determined by the Folin-Lowry method at 660 nm using tyrosine as the calibration standard [23]. Because this approach quantifies disappearance of soluble protein rather than directly measuring protein bound to the carrier, values are reported as apparent immobilization efficiency. Immobilization efficiency and retained activity were determined in 3 independently prepared PAD-serratiopeptidase batches, and each analytical measurement was performed in technical triplicate. Batch-to-batch reproducibility was evaluated by comparing immobilization efficiency and activity values across the independent preparations.

$$\text{Immobilization efficiency (\%)} = \frac{(C_0 - C_{res})}{C_0} \times 100$$

where C_0 is the initial protein concentration and C_{res} is the residual protein concentration in the combined supernatant and wash fractions.

FTIR spectroscopy

Fourier-transform infrared (FTIR) spectra of native dextran, PAD, free serratiopeptidase, and PAD-immobilized serratiopeptidase were recorded on a Bruker Vector-22 FTIR spectrometer. Samples were prepared as KBr pellets and scanned over 400 - 4,000 cm^{-1}. The aldehyde-associated C=O band of PAD, amide bands of serratiopeptidase, and imine-associated C=N region of the conjugate were analyzed. Relative changes in selected band intensities were interpreted semi-quantitatively as supportive evidence of oxidation and enzyme attachment. FTIR was not considered sufficient alone to determine covalent-bond density, enzyme orientation, surface morphology, particle size, or zeta potential. Advanced techniques such as SEM, TEM, XPS, NMR, zeta potential, or particle-size analysis were not included because the present study focused on soluble/hydrated PAD conjugate preparation and biochemical performance; these methods are

proposed for future structural validation.

Proteolytic activity assay

Proteolytic activity was measured using a modified casein digestion assay based on colorimetric detection of soluble peptide products [24]. Free or immobilized serratiopeptidase was incubated with 1.0% (w/v) casein prepared in 50 mM phosphate buffer (pH 7.0). Unless otherwise indicated, 0.5 mL of enzyme preparation was mixed with 0.5 mL of substrate solution and incubated at 37 °C for 10 min. The reaction was stopped by adding 1.0 mL of 10% trichloroacetic acid. After centrifugation to remove precipitated protein, the soluble peptide fraction was reacted with Folin-Ciocalteu reagent, and absorbance was measured at 660 nm. Tyrosine and tryptophan standards were used to construct calibration curves for colorimetric response. One unit of proteolytic activity was defined as the amount of enzyme releasing 1 umol of tyrosine equivalents per minute under the assay conditions. Activities of free and immobilized preparations were compared after normalization to nominal protein content. The overall assay sequence is summarized in **Figure 3**. Each assay was performed using 3 independently prepared samples with technical triplicate measurements.

Figure 3 Workflow for the casein-based proteolytic activity assay used to evaluate free and PAD-immobilized serratiopeptidase. Enzyme preparations were incubated with casein substrate, the reaction was stopped with trichloroacetic acid, and soluble peptide products were quantified using Folin-Ciocalteu detection against tyrosine/tryptophan standards. The assay output was expressed as tyrosine-equivalent activity under the stated reaction conditions, enabling direct comparison of apparent proteolytic performance.

Experimental controls, kinetic analysis, stability design and statistical analysis

All activity measurements included substrate blanks, carrier blanks, and heat-inactivated enzyme controls. For immobilized samples, washing fractions were analyzed to estimate residual soluble protein, and activity values were corrected for carrier blanks. Apparent kinetic parameters were estimated by nonlinear regression of initial reaction rate versus substrate concentration using the Michaelis-Menten equation, and Lineweaver-Burk analysis was used only as a visual check of fitting consistency [25]. Because casein is a heterogeneous protein substrate and the exact concentration of catalytically accessible immobilized enzyme was not directly determined, Km and Vmax are reported as apparent parameters and kcat was not calculated. pH stability was evaluated after 24 h incubation at pH 4.0 - 9.0 at 25 °C. Thermal stability was evaluated after 1 h incubation at 25 - 60 °C. Storage stability was evaluated during refrigerated storage at 4 °C for up to 60 days. Results are reported as mean +/− SD from 3 independent measurements unless otherwise stated. Normality was checked using the Shapiro-Wilk test. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test in OriginPro 2021 software, and differences were considered significant at $p < 0.05$. Where exact p-values were not available from the original data export, statistical significance is reported as $p < 0.05$ or $p < 0.01$.

Results and discussion

Structural confirmation of immobilization

Periodate oxidation of dextran generated a reactive aldehyde-containing carrier suitable for coupling with serratiopeptidase. In the FTIR spectrum of PAD, the appearance of a band around 1,730 cm^{-1} was assigned to aldehyde C=O stretching, which is consistent with oxidation of the polysaccharide backbone. After immobilization, the aldehyde-associated band decreased in intensity and a signal in the 1,630 - 1,650 cm^{-1} region became more pronounced. This region overlaps with imine C=N vibrations and protein amide I absorption. The conjugate spectrum also retained amide I and amide II features attributable to serratiopeptidase and C−O/C−O−C bands characteristic of dextran, indicating the coexistence of enzyme and polysaccharide components in the immobilized preparation. Together with hydroxylamine titration of PAD and indirect protein loading data, the FTIR results support successful preparation of the PAD-serratiopeptidase conjugate. Representative spectra are shown in **Figure 4**.

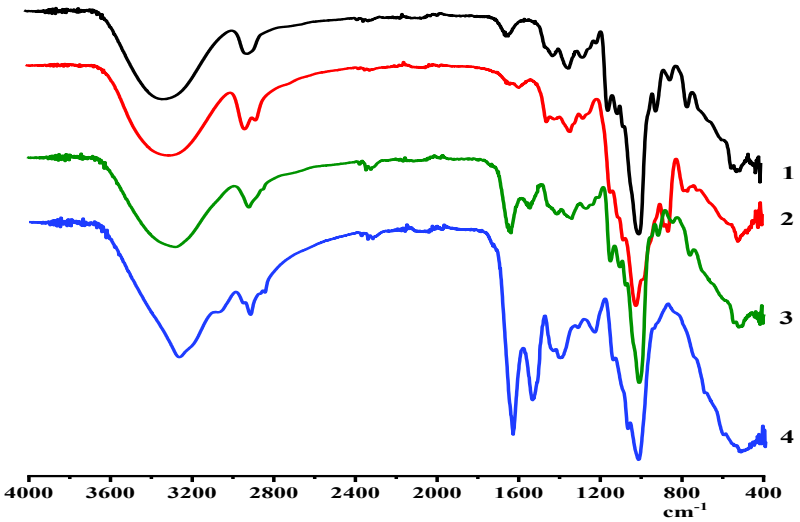


Figure 4 FTIR spectra of native dextran, PAD, PAD-immobilized serratiopeptidase, and free serratiopeptidase used to support carrier oxidation and enzyme attachment. The PAD spectrum shows an aldehyde-associated C=O band near 1,730 cm^{-1}, while the conjugate retains protein amide features and polysaccharide C-O/C-O-C bands. The decrease in aldehyde-associated intensity together with the more pronounced 1,630 - 1,650 cm^{-1} region is consistent with Schiff base-associated coupling, although FTIR is interpreted as supportive rather than definitive structural proof.

Overall, the FTIR data provide initial structural support for immobilization of serratiopeptidase on PAD and preservation of the major polysaccharide framework. However, FTIR alone cannot quantify the number of enzyme-carrier bonds, determine immobilized enzyme orientation, or fully characterize secondary-structure changes. For this reason, the structural interpretation in this study is limited to evidence of PAD oxidation, protein incorporation, and probable Schiff base-associated coupling. More definitive confirmation would require complementary techniques such as elemental analysis, XPS, NMR, amino-group consumption assays, or microscopic/morphological characterization.

Optimization of immobilization conditions

Enzyme-to-carrier ratio: The apparent immobilization efficiency depended strongly on the enzyme:PAD ratio and is presented as mean +/- SD from 3 independent immobilization batches. Increasing the relative amount of carrier increased apparent binding, reaching approximately 92% at an enzyme:carrier ratio of 1:6 (w/w). However, the highest binding yield did not correspond to the best functional outcome. At excessive carrier proportion, retained proteolytic activity decreased, which may reflect restricted enzyme mobility, steric effects, or lower substrate accessibility. The best functional compromise was observed at a ratio of 1:3, where apparent immobilization efficiency remained about 85% - 90% while activity was better preserved. These data were obtained using a one-factor-at-a-time optimization approach and should therefore be interpreted as empirical optimization rather than as a full factorial or response-surface model.

The proposed explanation involving multipoint attachment and steric restriction is consistent with established immobilization principles [4,5,22], but it was not directly verified by structural or molecular modeling in the present work. Therefore, the ratio of 1:3 is presented as the experimentally selected operating condition for this PAD-serratiopeptidase system, not as a universal optimum.

pH of immobilization

The pH of the coupling reaction strongly influenced immobilization outcomes (**Figure 5**).

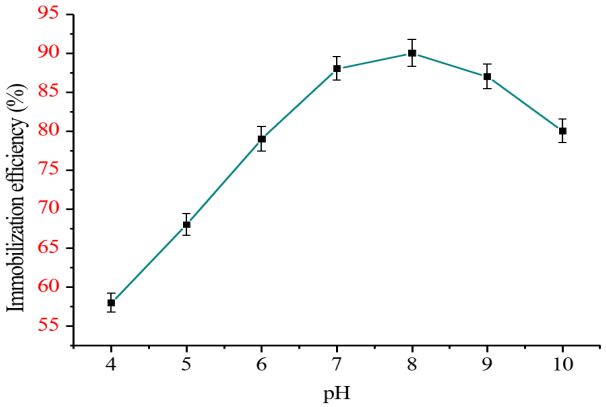


Figure 5 Effect of coupling pH on the apparent immobilization efficiency of serratiopeptidase on PAD. Efficiency increased from acidic to near-neutral conditions and reached the highest values around pH 6.5 - 7.5, where amino groups are sufficiently reactive while enzyme structure is better preserved. The decrease under more acidic or alkaline conditions indicates that both amino-group protonation and enzyme/imine stability influence the immobilization outcome.

Figure 5 illustrates the influence of pH on the apparent immobilization efficiency of serratiopeptidase on PAD. Efficiency increased progressively from acidic to near-neutral conditions, with the highest values observed at pH 6.5 - 7.5, reaching approximately 90%. At pH 4 - 5, immobilization efficiency was markedly lower, which is consistent with reduced nucleophilicity of protonated amino groups. At pH values above 8, efficiency decreased slightly, which may be related to enzyme destabilization, side reactions, or reduced stability of imine linkages.

These results support the selection of near-neutral coupling conditions as a compromise between chemical reactivity of amino groups and preservation of serratiopeptidase activity. The broad pH screening was used only to identify a practical coupling window; it was not intended to imply that serratiopeptidase remains structurally unchanged throughout the entire pH range.

Therefore, pH 6.5 - 7.5 was selected for subsequent experiments because it produced high apparent immobilization efficiency while minimizing exposure to strongly destabilizing conditions.

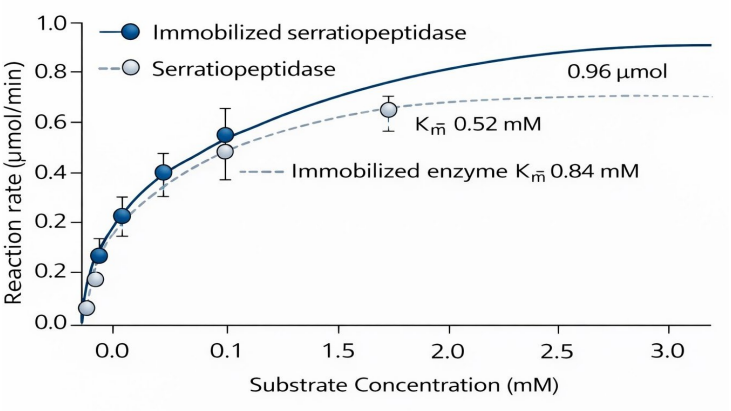


Figure 6 Apparent substrate-concentration response of free and PAD-immobilized serratiopeptidase in the casein digestion assay. The curves compare initial reaction rate as substrate concentration increases and were used to estimate apparent Km and Vmax values by nonlinear regression. Because casein is a heterogeneous macromolecular substrate and the accessible immobilized enzyme fraction was not directly measured, the parameters should be interpreted as functional, assay-specific kinetic descriptors rather than intrinsic catalytic constants.

Figure 6 presents the apparent activity response of free and immobilized serratiopeptidase toward increasing casein substrate concentration. Nonlinear regression provided apparent kinetic constants

summarized in **Table 1**. The free enzyme showed an apparent Km of approximately 0.52 mM and an apparent Vmax of approximately 0.72 umol/min, whereas the PAD-immobilized preparation showed an apparent Km of approximately 0.84 mM and an apparent Vmax of approximately 0.96 umol/min. The higher apparent Km of the immobilized preparation may indicate diffusional resistance or reduced substrate accessibility within the hydrated PAD environment, while the higher apparent Vmax under the tested conditions is consistent with improved dispersion and reduced short-term inactivation. Because the concentration of catalytically accessible immobilized enzyme was not directly determined, kcat was not calculated.

Table 1 Apparent kinetic parameters of free and PAD-immobilized serratiopeptidase estimated from casein digestion assays.

Preparation	Apparent Km (mM)	Apparent Vmax (umol/min)	kcat	Interpretation
Free serratiopeptidase	0.52	0.72	Not calculated	Lower apparent Km, but lower plateau activity under assay conditions
PAD-immobilized serratiopeptidase	0.84	0.96	Not calculated	Higher apparent Vmax; possible diffusion/accessibility effects should be considered

These values should be interpreted cautiously because casein is a heterogeneous macromolecular substrate and the immobilized preparation may be influenced by substrate diffusion, carrier hydration, and enzyme distribution inside or along the PAD matrix. Therefore, the kinetic analysis supports the practical conclusion that PAD immobilization improves apparent and reproducible proteolytic performance, but it does not establish an intrinsic increase in catalytic turnover.

A more detailed kinetic study using soluble chromogenic or fluorogenic peptide substrates would be required to separate true catalytic effects from diffusion, accessibility, and stabilization effects.

Accordingly, the kinetic data are used here to define a functional operating profile for the PAD-serratiopeptidase preparation rather than to claim a complete mechanistic Michaelis-Menten characterization.

Proteolytic activity: Free vs. immobilized enzyme

As shown in **Figure 7**, proteolytic activity assays revealed a more proportional concentration-activity relationship for PAD-immobilized serratiopeptidase than for the free enzyme. The free enzyme showed a plateau at higher nominal concentrations, which may be caused by aggregation, self-interaction, autoproteolysis, or partial inactivation during handling. In contrast, the immobilized preparation showed a more linear response within the tested concentration interval. At equivalent nominal protein contents, the immobilized enzyme exhibited approximately 2.1 - 3.1-fold higher apparent activity than the free enzyme. This finding is interpreted as an apparent functional enhancement under assay conditions, not as proof of an increased intrinsic catalytic constant.

The practical significance of the more proportional dose-response is that enzyme amount can be linked more reliably to expected proteolytic output. In pharmaceutical formulation, such predictability is important for reproducible batch potency and dose adjustment; in industrial biocatalysis, it supports process control, scale-up, and consistent substrate conversion. Nevertheless, possible mass-transfer limitations, substrate diffusion within the hydrated carrier environment, and the exact amount of catalytically accessible immobilized enzyme were not directly quantified and should be considered when translating the present system to preparative applications.

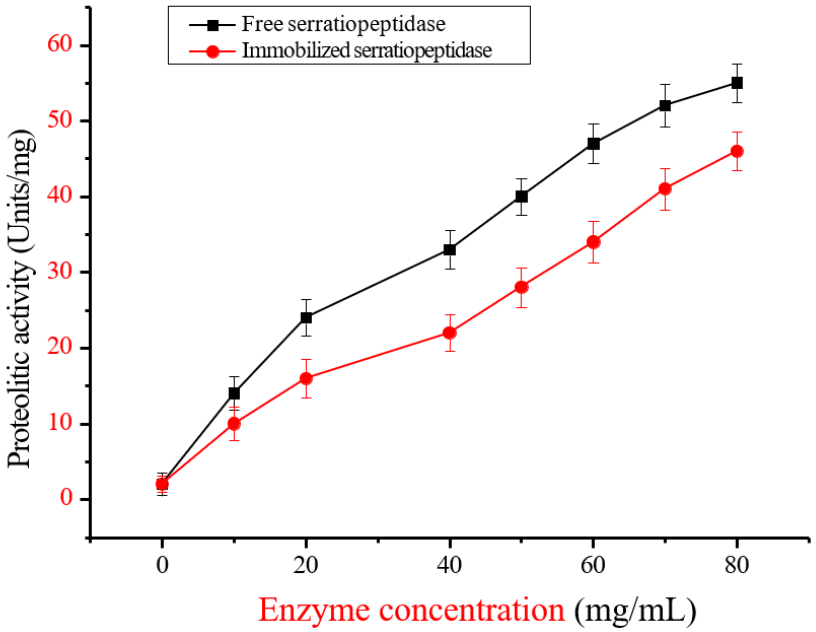


Figure 7 Effect of nominal enzyme concentration on proteolytic activity of free and PAD-immobilized serratiopeptidase. The immobilized preparation showed a more proportional concentration-activity response across the tested range, whereas the free enzyme plateaued at higher concentrations. This pattern supports the use of PAD immobilization for more reproducible enzyme dosing, while the apparent activity gain should be interpreted in the context of possible aggregation, autoproteolysis, and carrier-related accessibility effects.

PAD-immobilized serratiopeptidase showed a more proportional increase in apparent activity with enzyme amount, whereas the free enzyme plateaued at higher concentrations. This behavior supports the practical advantage of PAD immobilization for reproducible enzyme dosing, especially when predictable activity is more important than maximizing nominal activity. The mechanistic origin of this effect requires further structural, leaching, and kinetic validation.

Stability evaluation of free and immobilized serratiopeptidase

pH stability

The stability of free and immobilized serratiopeptidase was assessed across pH 4.0 - 9.0 by measuring residual activity after incubation in buffers of varying pH. The free enzyme retained significant activity mainly within a relatively narrow range around pH 6.5 - 7.5 and lost more than 50% of activity at pH < 5.5 or > 8.0. In contrast, the PAD-immobilized enzyme maintained more than 80% of its initial activity across pH 6.0-8.0 and retained higher residual activity than the free enzyme at mildly acidic and mildly alkaline pH values. These results demonstrate improved pH tolerance under the tested conditions. The proposed role of PAD in maintaining local hydration and limiting conformational shifts is consistent with immobilization literature [4,5,21,22], but direct microenvironmental pH or conformational measurements were not performed.

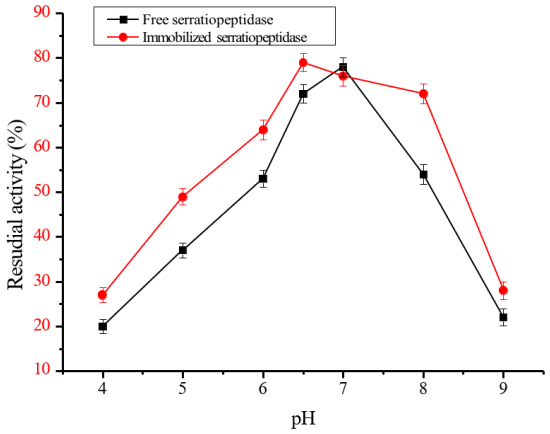


Figure 8 pH stability profiles of free and PAD-immobilized serratiopeptidase after 24 h incubation at pH 4.0 - 9.0 and 25 °C. Residual activity remained higher for the immobilized preparation, especially across the near-neutral range of pH 6.0 - 8.0, where it retained more than 80% activity. The improved profile indicates functional pH stabilization by the PAD matrix, although local pH buffering and conformational effects were not directly measured.

Figure 8 shows that the immobilized enzyme retained higher residual activity than free serratiopeptidase across the tested pH range. The data support improved functional pH stability, while the precise contribution of carrier hydration, local pH buffering, and conformational restriction remains to be verified by complementary techniques.

Thermal stability

Thermal stability was examined by incubating enzyme samples at 25 - 60 °C for 1 h and measuring residual activity, as shown in **Figure 9**. Free serratiopeptidase was rapidly inactivated above 40 °C; at 50 °C it retained less than 20% of initial activity after 1 h. In contrast, PAD-immobilized serratiopeptidase retained approximately 70% of its activity after the same treatment at 50 °C and approximately 50% at 60 °C. These data show a substantial improvement in single-time-point thermal resistance. However, thermal half-life (t1/2), activation energy of deactivation, and denaturation parameters were not determined; therefore, the thermal analysis is comparative rather than mechanistically complete.

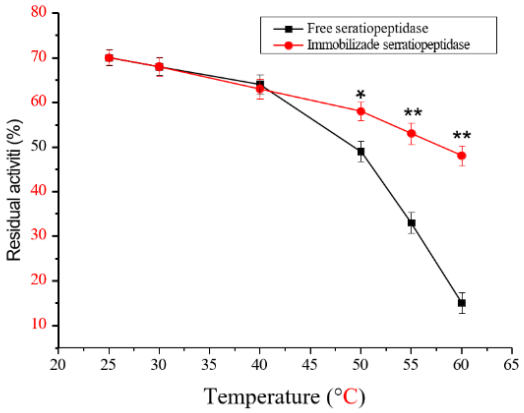


Figure 9 Thermal stability of free and PAD-immobilized serratiopeptidase after 1 h exposure to 25 - 60 °C. Residual activity of the free enzyme decreased sharply above 40 °C, whereas the immobilized preparation retained about 70% activity at 50 °C and about 50% at 60 °C. Asterisks indicate statistically significant differences between free and immobilized preparations (*$p < 0.05$, **$p < 0.01$; mean +/− SD, n = 3), supporting improved thermal resistance under this single-time-point assay.

The observed improvement is consistent with reports that covalent immobilization can reduce conformational mobility and aggregation under thermal stress [4,5,22]. Nevertheless, the present data do not directly distinguish between conformational rigidification, reduced autoproteolysis, improved dispersion, or carrier-associated substrate accessibility. Compared with TiO2-based serratiopeptidase systems, which reported improved thermal stability and antibacterial activity after nanoparticle bioconjugation [9], the PAD system emphasizes a hydrophilic polysaccharide carrier and proportional proteolytic response rather than multifunctional inorganic nanoparticle behavior.

Storage stability

Storage stability was evaluated by storing both free and immobilized enzyme at 4 °C and periodically measuring activity for up to 60 days. Free serratiopeptidase lost more than 50% of its initial activity within 30 days, whereas the immobilized enzyme retained approximately 85% of initial activity over the same period. After 60 days, the immobilized preparation retained more than 70% activity, while the free enzyme retained less than 20%. These results indicate that PAD immobilization improves storage stability under refrigerated conditions.

The improved storage behavior may reflect reduced enzyme aggregation, partial protection against autoproteolysis, and stabilization within the hydrated polymer matrix. However, this experiment evaluated residual activity during static storage rather than operational stability during repeated cycles. Direct leaching tests, wash-challenge experiments, protein release during long-term incubation, substrate-specificity testing, and multi-cycle activity recovery were not performed and should be included in future applied studies.

Proposed mechanistic interpretation and study limitations

The enhanced apparent activity and stability of PAD-immobilized serratiopeptidase can be interpreted through a working mechanism consistent with the scheme and experimental profiles shown in **Figures 2**, **5** and **9**. Free serratiopeptidase may undergo concentration-dependent self-interaction, aggregation, or autoproteolysis, leading to a plateau in activity at higher nominal enzyme concentrations. PAD immobilization can spatially separate enzyme molecules, provide a hydrated polysaccharide microenvironment, and reduce large conformational fluctuations through covalent anchoring. These effects may explain the more proportional concentration-activity response and higher residual activity after pH, thermal, and storage stress. However, this mechanism remains a hypothesis supported by functional data and immobilization literature [4,5,21,22], not by direct conformational spectroscopy or molecular modeling.

It is also important to recognize that immobilization can reduce activity if the enzyme is attached through residues near the active site, if multipoint attachment becomes excessive, or if diffusion of a macromolecular substrate such as casein becomes limiting. The selected PAD oxidation degree (approximately 25% - 30%) and enzyme:carrier ratio (1:3) appear to provide a favorable empirical compromise between binding efficiency and retained activity. Future work should determine kinetic constants using soluble model substrates, quantify leaching and reusability, evaluate substrate specificity, and assess structural changes by CD, fluorescence, XPS/NMR, SEM/TEM, or other appropriate techniques. For translational use, additional pharmaceutical formulation testing and application-specific *in vitro* or *in vivo* validation will be required.

Comparison with literature data

To contextualize the findings without overgeneralization, **Table 2** compares the PAD-serratiopeptidase system with closely related serratiopeptidase/protease immobilization concepts and relevant dextran-aldehyde chemistry.

Table 2 Critical comparison of the PAD-serratiopeptidase system with selected closely related immobilized protease and dextran-aldehyde systems.

Enzyme / system	Carrier / coupling strategy	Standardized quantitative outcome	Main advantage	Main limitation / caution	Ref.
Serratiopeptidase	PAD; Schiff base coupling under mild aqueous conditions	Apparent immobilization efficiency about 85% - 90%; > 80% residual activity at pH 6 - 8; about 70% residual activity after 1 h at 50 °C; about 85% residual activity after 30 days at 4 °C	Hydrophilic polysaccharide carrier; proportional concentration-activity response; simple preparation	Covalent bonding supported but not fully quantified; no direct leaching, reuse-cycle, or advanced structural data	This work
Serratiopeptidase	Carboxylated TiO_2 nanoparticles; EDC/NHS coupling	Optimal specific activity reported at activation pH 5.1/coupling pH 5.5; 63.61% residual activity after 48 h at 37 °C vs 39.31% for free enzyme; 2-fold higher antibacterial activity at 1,000 ug/mL	Direct serratiopeptidase benchmark; multifunctional nanoparticle system	Requires inorganic support functionalization; main emphasis on antibacterial activity rather than proportional proteolytic dosing	[9]
Bromelain and related proteases	Polysaccharide, hydrogel, and nanomaterial supports	Reported stability and handling improvements are support-dependent and vary across systems	Useful protease benchmark for carrier-dependent immobilization behavior	Different enzyme; quantitative comparison is indirect unless identical assay conditions are used	[8]
Covalently immobilized enzymes/proteases	Aldehyde, glyoxyl, epoxide, and related reactive supports	Multipoint attachment often increases stability, but excessive attachment can reduce activity	Mechanistic framework for balancing stabilization and retained activity	General immobilization literature; not serratiopeptidase-specific	[5,22]
Dextran-aldehyde enzyme systems	Oxidized dextran as spacer/crosslinking polymer or enzyme microenvironment modifier	Dextran aldehyde can stabilize enzymes and modulate local microenvironment depending on oxidation and coupling conditions	Closest carrier-chemistry precedent for PAD	Most evidence concerns other enzymes; direct PAD-serratiopeptidase data remain limited	[21]

The comparison indicates that PAD-serratiopeptidase occupies a distinct position among immobilized protease systems. TiO_2-serratiopeptidase bioconjugates provide a direct enzyme-specific benchmark and include nanoparticle characterization and antibacterial testing, but they require inorganic support functionalization and are oriented toward antimicrobial functionality [9]. PAD, in contrast, uses a water-soluble polysaccharide carrier and mild Schiff base chemistry, making it suitable for stabilization and proportional proteolytic dosing. Its main limitation is the present absence of advanced structural, leaching, and reuse-cycle data. Therefore, PAD should be viewed as a promising but still incompletely characterized platform rather than as a universally superior carrier.

Conclusions

Polyaldehyde dextran obtained by controlled periodate oxidation of dextran was evaluated as a hydrophilic polysaccharide carrier for covalent immobilization of serratiopeptidase. Under the experimental conditions used here, an enzyme-to-carrier ratio of 1:3 and coupling pH of 6.5 - 7.5 provided a favorable balance between apparent immobilization efficiency (approximately 85% - 90%) and retained proteolytic activity. FTIR spectroscopy, aldehyde-content determination, and indirect protein loading supported formation of the PAD-serratiopeptidase preparation, although covalent-bond density and enzyme orientation were not directly determined.

The principal scientific contribution is the demonstration that PAD immobilization can generate a serratiopeptidase preparation with more proportional concentration-activity behavior and improved residual activity after pH, thermal, and refrigerated storage challenges. The system is best positioned at this stage as a biochemical and biotechnological enzyme-stabilization platform; pharmaceutical application remains possible but requires additional formulation, safety, potency, and efficacy studies.

The main limitations are the absence of advanced structural characterization, direct leaching assessment, repeated-use/operational stability testing, substrate-specificity evaluation, and *in vitro* or *in vivo* validation. Future work should address these points and compare PAD with chitosan, alginate, silica, and inorganic nanoparticle supports under standardized conditions. Within these limits, PAD-based immobilization represents a practical approach for preparing stabilized serratiopeptidase systems with controllable proteolytic output.

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Declaration of Generative AI in Scientific Writing

The authors declare that generative tools were used exclusively for language editing and grammatical refinement during manuscript preparation. These tools were not involved in the generation of scientific content, data analysis, or interpretation of the results. All research findings, analyses, and conclusions are solely the responsibility of the authors, who have fully reviewed and approved the final manuscript.

CRediT Author Statement

Nargiza Abduvasitova: Formal analysis; Data curation. **Shavkat Shomurotov:** Data curation; Writing - Original draft preparation. **Gulnora Tillayeva:** Writing - review & editing; Methodology; Conceptualization. **Umida Tillayeva:** Methodology; Software. **Olimov Nemat:** Visualization; Investigation. **Fazliddin Jalilov:** Methodology; Software. **Feruza Jalilova:** Visualization; Investigation.

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