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RESEARCH ARTICLE

AN IMPROVED AND INTEGRATED MEMBRANE-BASED CLARIFICATION AND CONTROLLED CRYSTALLIZATION STRATEGY FOR THE EFFICIENT PURIFICATION OF PHARMACEUTICAL-GRADE SERRATIOPEPTIDASE

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ABSTRACT

Serratiopeptidase, a zinc-dependent metalloprotease produced by Serratia marcescens, is widely recognized for its therapeutic applications, notably in anti-inflammatory and fibrinolytic treatments. Despite its pharmaceutical significance, efficient large-scale purification remains a challenge due to complex downstream processing requirements. This study presents a systematic approach to optimize the downstream processing of serratiopeptidase, focusing on membrane-based clarification and controlled crystallization. The fermentation broth was first clarified using microfiltration to remove biomass and insoluble particulates, followed by ultrafiltration to concentrate the enzyme and reduce process volume while preserving enzymatic activity. Crystallization parameters—including pH, temperature, and solvent system—were individually evaluated to enhance product yield and purity. The optimal conditions were identified as pH 6.0, a temperature range of 0–5°C, and acetone as the anti-solvent, resulting in a maximum yield of 62.86% and activity of 2535 units/mg, meeting pharmacopeial standards. The integrated process significantly improved recovery, reproducibility, and scalability, offering a robust purification strategy suitable for pharmaceutical production. This work provides valuable insights for developing efficient and compliant purification platforms for industrial-scale enzyme manufacturing.

KEYWORDS

Serratiopeptidase, Serratia marcescens, Serralysin family, Downstream processing, Enzyme purification, Process optimization, Pharmaceutical enzymes, Enzyme recovery, Anti-solvent precipitation, Enzyme assay

INTRODUCTION

Serretiopeptidase, also known as Serratia E-15 protease, serralysin, serrapeptase, serratiapeptase, serratia peptidase, serratio peptidase, or serrapeptidase, is an extracellular zinc-dependent metalloprotease primarily produced by the enterobacterium *Serratia* sp. E-15, now known as *Serratia marcescens* ATCC 21074. The enzyme was originally identified in the intestinal tract of the silkworm (Bombyx mori), where it facilitates the degradation of cocoon proteins during metamorphosis, highlighting its strong proteolytic capability (1). Owing to its broad substrate specificity and high catalytic efficiency, serretiopeptidase has been extensively studied and widely used for its anti-inflammatory, anti-edematous, fibrinolytic, and analgesic properties (2, 3).

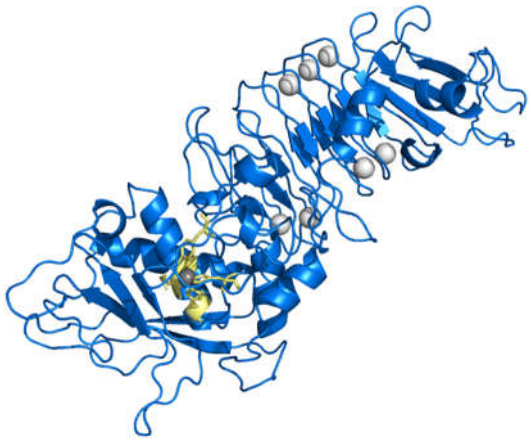

Figure-1: Crystal structure of Serralysin with co-ordinated zinc and calcium (4).

Structurally, serretiopeptidase belongs to the serralysin family (EC 3.4.24.40) and requires zinc at its active site for catalytic activity. The enzyme typically exhibits a molecular weight in the range of 45-60 kDa, depending on the microbial strain, fermentation conditions, and purificati on strategy employed(5, 6). Its optimal activity under mild alkaline conditions and physiological temperatures has contributed to its long-standing clinical use, particularly in postoperative inflammation, respiratory disorders, and musculoskeletal conditions (7).

With increasing pharmaceutical demand, considerable research efforts have been directed toward improving the production and downstream processing of serretiopeptidase. The enzyme is secreted extracellularly during submerged fermentation, which simplifies recovery compared to intracellular proteins; however, downstream purification remains a major challenge due to the presence of host proteins, media components, pigments, and proteolytic impurities (8, 9). Traditional purification strategies such as ammonium sulfate precipitation and chromatographic techniques have been widely reported, but are often associated with high operational costs, limited scalability, and significant product loss (10).

Downstream processing challenges are further compounded by the intrinsic sensitivity of serretiopeptidase to pH, temperature, and solvent exposure. Improper clarification can lead to membrane fouling, while harsh purification conditions may cause enzyme denaturation or loss of activity (11). Membrane-based operations such as microfiltration and ultrafiltration have therefore gained attention as effective pre-purification steps, enabling efficient biomass removal, volume reduction, and partial impurity clearance while preserving enzyme integrity (5).

Among alternative purification approaches, controlled crystallization has emerged as a promising technique due to its simplicity, cost-effectiveness, and suitability for large-scale enzyme recovery. However, crystallization of enzymes presents unique challenges, including control of nucleation, crystal morphology, and reproducibility, which are strongly influenced by pH, temperature, and solvent system (1, 9). Variability in these parameters often leads to inconsistent yield and purity, underscoring the need for systematic optimization.

The isolation of serratiopeptidase from fermentation broth involves several key steps:

- Separation of cells: Microfiltration to remove microbial cells, cell debris, and other insoluble particulates. This initial clarification step is critical in downstream processing, as it prevents fouling of subsequent membranes and minimizes product loss. Microfiltrati on enabled efficient separation of the biomass while allowing the extracellular serretiopeptidase to remain in the permeate, thereby improving process throughput and enzyme recovery.

- Volume Concentration: Ultrafiltration to concentrate serretiopeptidase and reduce the overall process volume. Ultrafiltration selectively retained the enzyme based on molecular size while permitting the passage of low-molecular-weight impurities, residual medium components, and salts. This step not only increased the enzyme concentration but also enhances product stability by maintaining mild operating conditions, thereby p reserving enzymatic activity. In addition, ultrafiltration improved downstream efficiency by producing a highly enriched feed suitable for final purification.
- Final Crystallization: Purification of serretiopeptidase was subsequently achieved through controlled crystallization, which served as the primary isolation technique. Crystallization was selected due to its simplicity, cost-effectiveness, and suitability for large-scale enzyme purification.

Regulatory standards further emphasize the importance of robust downstream processing. The Indian Pharmacopeia (IP) (12) specify stringent requirements for enzyme activity for pharmaceutical preparations containing proteolytic enzymes. Meeting these regulatory

expectations necessitates purification processes that are not only efficient but also reproducibl e, scalable, and compliant with pharmacopeial quality attributes.

Despite extensive research on serretiopeptidase production, relatively fewer studies have focused on addressing downstream processing challenges in a systematic manner, such as are-

- Crystallization-based purification strategies
- Variability in reported yields
- Enzyme activity as per pharmacopeial standards
- Process robustness highlights the need for detailed evaluation of critical physicochemical parameters influencing enzyme isolation.

Therefore, the present study aims to investigate and optimize downstream processing of serretiopeptidase, with particular emphasis on membrane-based concentration and controlled crystallization, to develop a scalable and pharmaceutically acceptable purification strategy.

MATERIALS & METHODS

Microorganism and cultivation conditions: The Serratiopeptidase that was made came from a mutant strain of *Serratia marcescens* ATCC 21074 that made a lot of it. The strain was kept at 25 degrees Celsius on a modified seed medium with the following composition (g/L): yeast extract, 5.8; casein, 4.5; bio peptone, 8.0; dextrose, 3.0; sodium chloride, 4.2. The fermentation process took place in a liquid medium for 48 ± 24 hours at 25 degrees Celsius with the same composition as seed medium. Growth was monitored by pH and microscopic examination, and active cultures exhibited characteristic pink pigmentation at pH 6.8 ± 0.2.

Isolation and Purification of Serretiopeptidase: After the completion of the fermentation process, the harvested broth was subjected to microfiltration by using 0.2µ ceramic membrane to remove microbial cells, cell debris, and other insoluble particulates. The clarified product solution was then processed by ultrafiltration of membrane of 10KDa to concentrate serretiopeptidase and reduce the overall process volume. pH of concentrated solution was adjusted to 5.0, 6.0, 7.0 or 8.0 and then cooled to 0-5, 10-15 or 20-25 degree Celsius. Lactose monohydrate was added in the product solution and mixed properly till dissolved. Then the Chilled solvent namely isopropyl alcohol (IPA), acetone, and ethanol was added in product solution to crystallize the product. The product wet cake was filtered and then dried at 25±5 degree Celsius under vacuum to obtained dry serratiopeptidase.

Identification: Identification and quantification of serretiopeptidase was carried out by UV absorbance. Serratiopeptidase activity depends on its ability to break down casein. Add 0.1 ml

of the assay sample to a substrate solution consisting of 0.75 ml of 1.0% w/v casein in 100 mM Tris-HCl, 1 mM MgCl2, and 2 mM PMSF at pH 8.0. Then incubated the mixture at 40 degree Celsius for 30 minutes. Then 0.75 ml of 10% (w/v) trichloroacetic acid (TCA) was added to precipitate the unhydrolyzed casein. After 15 minutes at 25 degree Celsius, filtered the reaction mixture. Then measured the absorbance of the clear solution at 280 nm. For the blank, 0.75 ml of 10% (w/v) TCA was added to 0.75 ml of the substrate solution and incubated at 40 degree Celsius for 30 minutes. Then add 0.1 ml of the assay solution and follow the same procedure as before. One unit of enzyme activity (EU) is the amount of enzyme that causes an increase of absorbance by 0.1 at 280 nm under the assay conditions.

RESULTS AND DISCUSSION

In the present study, the crystallization conditions for serretiopeptidase purification were systematically optimized by evaluating the effects of pH, temperature, and solvent system. These parameters play a critical role in determining product isolation pattern, yield, and product activity.

Effect of pH on Crystallization: Post-ultrafiltration, the resultant concentrated mass is subject to pH adjustment. The influence of pH on crystallization behavior was examined over the range of 5.0 to 8.0. At pH 6.0, uniform and well-defined product isolation was observed, resulting in high yield and purity. In contrast, crystallization at pH 5.0, 7.0, and 8.0 leads to poor product isolation, poor yield, and low purity.

Table-01: Effect of pH on Yield and Activity

Sr. No.	pH of Solution	Yield (%)	Activity (Units/mg)
1.	5.0	58.37%	2850.3 units/mg
2.	**6.0**	**62.86%**	**2535.0 units/mg**
3.	7.0	52.30%	2020.0 units/mg
4.	8.0	40.45%	1980.0 units/mg

Effect of Temperature on Crystallization: Temperature significantly influenced the isolation pattern and yield. The influence of temperature on crystallization behavior was examined over the range of 5.0 to 25.0 degrees Celsius. At a temperature of 0-5 degrees Celsius, optimum product isolation was observed, resulting in high yield and purity. In contrast, crystallization at temperature 10-15 or 20-25 degrees Celsius leads to poor yield and quality.

Table-02: Effect of Temperature on Yield and Activity

Sr. No.	pH of Solution	Yield (%)	Activity (Units/mg)
1.	**0.0 – 5.0**	**62.86%**	**2535.0 units/mg**
2.	10.0 – 15.0	48.35%	1985.0 units/mg
3.	20.0 – 25.0	22.67%	1568.0 units/mg

Effect of Solvent Systems on Crystallization: The impact of different organic solvents on crystallization efficiency was also evaluated. Among the tested solvents (isopropyl alcohol, acetone, and ethanol), acetone crystallizes well-defined amount of solid material with high recovery and purity, indicating superior anti-solvent properties for serretiopeptidase crystallization. Isopropyl alcohol (IPA) and ethanol result in lower recovery and moderate purity.

Table-03: Effect of Solvent on Yield, and Activity

Sr. No.	pH of Solution	Yield (%)	Activity (Units/mg)
1.	**Acetone**	**62.86%**	**2535.0 units/mg**
2.	Isopropyl Alcohol	57.35%	2221.0 units/mg
3.	Ethanol	25.30%	2189.0 units/mg

CONCLUSION

Based on the combined evaluation of pH, temperature, and solvent effects, the optimal cryst allization conditions were identified as pH of concentrated solution to adjusted to 6.0, then cooled to 0 – 5 degree Celsius and acetone to be use as crystallizing solvent. Under these conditions, serratiopeptidase was obtained with a maximum yield of approximately 62.86%, along with required activity assay (2535 units/mg) as per the pharmacopeial standards. These optimized parameters provide a robust and scalable approach for efficient downstream purification of serretiopeptidase.

Broth Clarification and Biomass Removal: Efficient clarification of the fermentation broth represented a major challenge due to high cell density, cell debris, and insoluble medium components. These factors increased the risk of membrane fouling and potential enzyme loss during separation. Optimization of microfiltration conditions were therefore essential to maintain adequate permeate flux while ensuring effective biomass removal without compromising serretiopeptidase recovery.

Enzyme Stability During Concentration: Serretiopeptidase is sensitive to variations in pH, temperature, and shear stress, which posed challenges during the concentration stage. Ultrafiltration required careful control of operating parameters to prevent enzyme activity loss while achieving efficient volume reduction and impurity removal.

Control of Crystallization Behavior: Crystallization posed significant challenges due to the strong dependence of serretiopeptidase solubility and nucleation on physicochemical conditions. Variations in pH, temperature, and solvent system led to inconsistent nucleation, irregular crystal morphology, and reduced yield. Precise control and systematic optimization of these parameters were required to obtain reproducible, well-defined crystals with acceptable purity.

This study successfully addressed key downstream processing challenges associated with the purification of serretiopeptidase by integrating membrane-based clarification and concentration with an optimized crystallization strategy. The optimized process enabled imp roved enzyme recovery, enhanced purity, and better reproducibility while minimizing product loss and overall process complexity. The developed downstream approach provides a practica l, scalable, and industry-relevant purification platform that complies with pharmaceutical quality requirements and supports efficient large-scale production of serretiopeptidase.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding this manuscript.

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