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Original Research Article

Comprehensive evaluation of enzymatic proteolysis for amlyoid reduction in macular amyloidosis by in vitro, in vivo and in silico methods

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ABSTRACT

Introduction: Skin diseases represent a significant burden on global healthcare systems, with protein deposition playing a pivotal role in their pathogenesis, including conditions such as macular amyloidosis. This study aimed to assess the proteolytic activity of the enzyme Papain on deposited protein, offering insights into potential therapeutic strategies.

Materials and Methods: Using a chicken model, the deposition of protein, a key component in skin diseases, was observed predominantly in the ileum. The dose in different concentrations was given to the ileum and observation was taken on alternative days. Dose of complex protein (albumin, beta lactalbumin, alpha-lactalbumin, Immunoglobulin 500mg/ml and 750mg /ml given respectively. Treatment with Papain demonstrated its ability to effectively break down the deposited protein. In invivo method the designated area of the animal's skin where the induction was occur to ensure proper application and observation were Shaved and cleaned. One vehicle control group, one disease group and one test group. The dosing was done by subcutaneous route. Dose of complex protein (albumin, beta lactalbumin, alpha-lactalbumin, Immunoglobulin) 500mg/ml and 750mg /ml were given . For Papaya Extract Dose of 500mg/ml and 750mg /ml were given to animals.

Result: In cheken ilium 5days after dosing, length of patch were observed for five days. In wistar rat after 4days the rats seem to develop hyper pigmented patches which were brown in color in disease group. Then the groups of Animal were injected with papain by SC route. Daily interval observations were done. On the 4th day treatment spot becomes reduced and it disappeared slowly. In silico score observed upto -8 which indicate positive binding affinity.

Conclusion: These findings underscore the potential of Papain as a promising candidate for the management of protein-related skin disorders, highlighting the importance of enzymatic intervention in mitigating their pathological manifestations.

1. Introduction

Skin conditions tend to occur more among the young generation. In India, most of the population suffers from serious skin conditions. Skin conditions can cause patients to experience severe emotional and psychological suffering that may be far worse than the physical effects.[1] Skin illness is one of the most common human diseases. Near about 30% and 70% of people are affected by skin disease, and even greater proportions are seen in at-risk subpopulations. It affects people of all ages and across all cultures.[2] Acne, Atopic Dermatitis (Eczema), Shingles (Herpes-Zoster), Hives (Urticaria), Sunburn, Contact-Dermatitis, Diaper Rash, and Rosacea these skin diseases are the most

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frequent. Amyloidosis is one of the autoimmune diseases in which the drug is not that much effective. Amyloidosis is a category of uncommon disorders marked by the extracellular deposition of abnormal amyloid proteins, which can affect one or more organ systems (systemic amyloidosis) or only one tissue site, such as the skin (localized amyloidosis).[3] Without systemic involvement, extracellular deposition of heterogenic amyloid proteins in the skin causes primary localized cutaneous amyloidosis (PLCA), macular amyloidosis (MA), and (primary localized cutaneous) nodular amyloidosis (NA), Lichen amyloidosis (LA) are three separate subtypes of primary localized cutaneous amyloidosis.[4] Asians, particularly Taiwanese and Indians have a higher probability of developing macular amyloidosis (MA). A prevalent kind of primary cutaneous amyloidosis (PCA) marked by scratchy brownish macules with rippling patterns.[5] Palitz and Peck published the first description of the macular variant of cutaneous amyloidosis in 1952. The age of onset for MA is between 21 and 50 years, and there is a pronounced female prevalence. Clinically, MA presents as poorly delineated hyperpigmented patches of greyish-brown macules with a rippled pattern, associated with deposition of amyloid material in the papillary dermis. The interscapular region is the area most frequently affected.[6] Primary cutaneous amyloidosis, a broad term, includes macular amyloidosis. Amyloid is accumulated in the top layer of the dermis in macular amyloidosis. Macular amyloidosis has been associated with friction and scratching, while the exact root cause is unknown.[7] Macular pigmented patches are usually found on the upper trunk of MA patients, particularly in the interscapular region, and the affected area is frequently itchy.[8] Since ancient times, people have made great use of plants as a source of medicine. In fact, according to the World Health Organisation, up to 80% of people still primarily use traditional remedies like herbs for their medications. Additionally, a number of modern medications are sourced from plants.[9] Natural products or compounds obtained from plants have a significant advantage over manufactured medications in terms of cost-effectiveness, ease of availability, and minor side effects.[10] The papaya, or Carica papaya Linn., is a member of the Caricaceae family and is well-known over the world for its healthful properties. Since the beginning of history, several portions of the papaya plant have been employed for therapeutic purposes.[11] There are numerous known medicinal uses for various papaya plant parts around the world, including the fruit, bark, roots, seeds, peel, pulp, and leaves.[12] The principal phytochemicals in papaya powder extract are Cystatin, papain, chymopapain, cyanogenic glucosides, tocopherol, and vitamin C, and is also rich in more than seventeen different amino acids and numerous important elements.[13] The enzyme papain, an endocytic plant cysteine protease, is generated from the papaya's (Carica papaya L.) latex. Papain is extracted by cutting the outer layer of unripe papaya and collecting and drying the latex that runs from the incision. Significant proteolytic activity of papain targets amide bonds, short-chain peptides, amino acid esters, and protein molecules.[14] Enzymatic debridement is facilitated by the proteolytic enzyme. Proteolytic enzymes include papain, pancreatic proteases, chymotrypsin, trypsin, bromelain, fungal proteases, and Serratia peptidase. The key objective of the current research study was to assess the proteolytic activity of the enzyme Papain on protein deposition while also observing protein deposition on the skin using chicken ileum.[15,16]

1.1. Pathogenesis of macular amyloidosis

Macular amyloidosis is a rare skin disorder that is characterized by the deposition of amyloid proteins in the skin. The exact mechanism of protein deposition is not fully understood, but it is believed to involve the following steps as shown in Figure 1 .

Figure 1: Steps involved in pathogenesis of Macular amyloidosis

1. Protein synthesis: Proteins are synthesized in the body, either in the skin or in other organs.
2. Misfolding: Some proteins may misfold or aggregate, meaning that they form abnormal structures that are not able to function properly.
3. Transport: The misfolded proteins are transported to the skin. E/F Gene leads to disturbance between α,ß globin synthesis.
4. Accumulation: The misfolded proteins accumulate in the dermis, which is the layer of skin below the outer layer (epidermis).
5. Binding to proteoglycans: The misfolded proteins bind to proteoglycans, which are large molecules found in the extracellular matrix of the skin.
6. Formation of amyloid fibers**:** The misfolded proteins then form amyloid fibers, which are insoluble and rigid structures that accumulate in the skin
7. Inflammation: The amyloid fibers may cause inflammation in the skin, leading to the characteristic appearance of macular amyloidosis.The exact triggers of protein misfolding and accumulation in macular amyloidosis are still under investigation. However, some studies have suggested that genetic factors may play a role in the development of the disorder
8. All above steps of formation of macular amyloid shown in Figure 2.

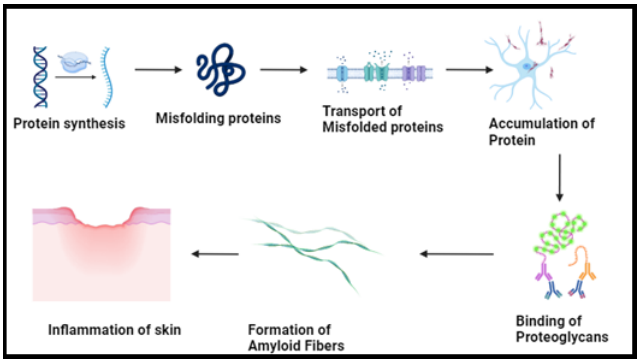


Figure 2: Steps involved in amyloid formation

1.2. Material

Chemicals: Complex protein mixture (albumin, beta lactalbumin, alpha-lactalbumin Immunoglobulin), Marketed Papaya extract powder as a source of Papain, Isolated Papain, Tyrode solution.

Tissue: Wistar Rat, Cock ileum

1.3. Experimental animals

Healthy adult Wistar rats (females), weighing 250–280 g, aged 5-7 weeks were used in this study. From Crystal Biological Solutions in Pune, India, animals were purchased. (2030/PO/RcBiBt/S/18/CPCSEA) and were housed in Dr. D. Y. Patil Institute Of Pharmaceutical Sciences And Research Pimpri, Pune.(198/PO/Re/S/2000/CPCSEA) In husk-lined polypropylene cages that were replaced every 24 hours 12:12 hour light/dark cycle with a 25± 3 °C ambient temperature. The caged rats had unrestricted access to reversible osmosis-purified water and a conventional diet pellet. Earlier to the trial, the rats spent a week becoming used to the laboratory environment. The Institutional Animal Ethics Committee accepted the experimental protocol (DYPIPSR/ IAEC/Sept/22-23/P-9) in compliance meeting the criteria set out by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

1.3.1. In silico study

In this study, an in silico docking approach was employed to assess the binding affinity between a target receptor and a ligand of interest. The molecular docking simulations were conducted using computational tools and software designed for such analyses. The three-dimensional structures of the receptor and ligand were prepared and optimized prior to docking. The docking simulations were performed with appropriate parameters and scoring functions to predict the binding conformation and affinity. Specifically, the docking studies were executed using established protocols, considering the receptor's active site and the ligand's binding mode. The docking scores were calculated to quantify the strength of interaction between the receptor and ligand. A docking score of -8 was obtained, indicating a favourable binding affinity between the ligand and receptor. Overall, the in silico docking method provided valuable insights into the molecular interactions, facilitating the understanding of ligand-receptor binding and potentially guiding further experimental studies.

Utilizing an in silico model, docking studies were conducted to evaluate the binding affinity between a specific ligand and its target receptor. The calculated docking score yielded a value of -5, and -8 respectively as shown in Figures 4 and 5 which indicating a moderate level of interaction between the ligand and receptor. This finding provides valuable insight into the potential molecular interactions and lays the groundwork for further investigations into the therapeutic efficacy and mechanism of action of the studied ligand.

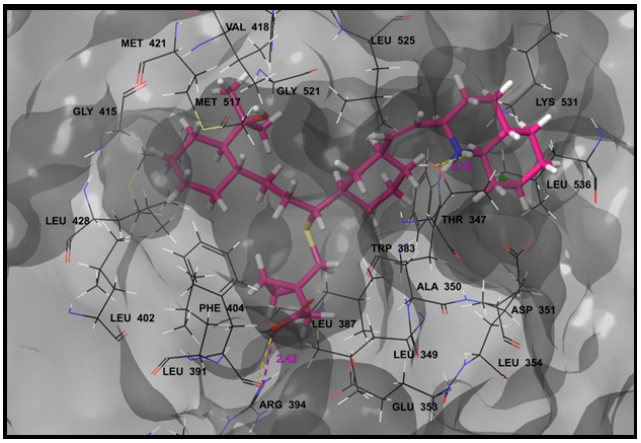


Figure 3: Molecular Docking with Score -5

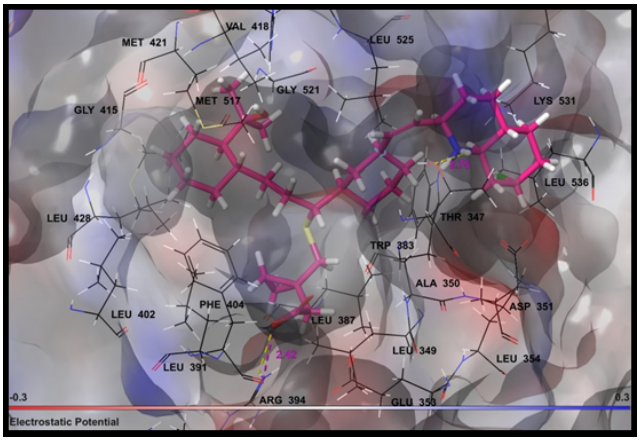


Figure 4: Molecular Docking with Score-8

1.4. Designing of experiment

1.4.1. In Vitro study

1.4.1.1. Method. The experiment was performed on cock ileum. Freshly isolated cock ileum was taken for the study. The dose in different concentrations was given to the ileum and observation was taken on alternative days. Dose of complex protein (albumin, beta lactalbumin, alpha-lactalbumin, Immunoglobulin 500mg/ml and 750mg /ml given respectively. For Papaya Extract dose of 500mg/ml and 750mg /ml were given as shown in Figure 5. Mixture of complex protein and Solution of Papaya shown inFigure 6. After dosing length of patch were observed for five days as shown in Table 1 and Figure 7.

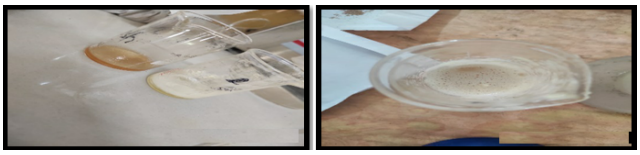


Figure 5: Mixture of complex protein and solution of papaya extract

Figure 6: Dosing of protein extract and dosing of papaya extract

Table 1: Response (length) of patches for five days

S.No.	Day 1	Day 2	Day 3	Day 4	Day 5
Response in patches(cm)	1.5 cm	2 cm	2.5 cm	3 cm	3.5 cm
Results (patches)					

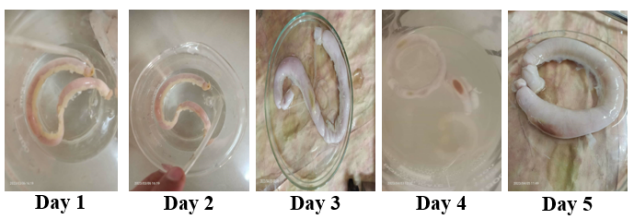


Figure 7: Response (length) of patches for five days (Table 1)

1.5. Experimental in Vivo induction of cutaneous amyloidosis

1.5.1. Method

The experiment was performed on Wistar rat. 3 groups of six rats in each were made. Administer anesthesia to the selected animals to ensure they are in a sedated state during the procedure. The designated area of the animal's skin where the induction was occur to ensure proper application and observation were Shaved and cleaned .The average weight of each rat was 200-250 gm. One vehicle control group, one disease group and one test group. The dosing was done by subcutaneous route. Dose of complex protein (albumin, beta lactalbumin, alpha-lactalbumin, Immunoglobulin) 500mg/ml and 750mg /ml were given. For Papaya Extract Dose of 500mg/ml and 750mg /ml were given to animals. After injection of protein in rat, groups of animals were given regular feed. After 4days the rats seem to develop hyper pigmented patches which are brown in color in disease group. Then the groups of Animal were injected with papain by SC route. The group of animals was given regular feed. Daily interval observations were done. On the 4th day treatment spot becomes reduced and it disappeared slowly.

2. Result and Discussion

The experiment was conducted on Wistar rats, divided into three groups, each consisting of six rats with an average weight ranging from 200 to 250 grams. The groups included a vehicle control group, a disease group, and a test group. Dosing was administered via the subcutaneous route. After the injection of protein into the rats, all groups were provided with regular feed. After four days, rats in the disease group began to exhibit hyperpigmented patches, characterized by a brown coloration. Subsequently, the animals in all groups were injected with papain via the subcutaneous route and continued to receive regular feed. Daily observations were conducted, revealing that by the fourth day of treatment, the hyper pigmented patches had noticeably reduced and eventually disappeared. In vitro observations shown in Figure 9 and In vivo observations shown in Figures 9 and 10.

3. Conclusion

Present research study yielded moderate success in detecting protein deposition within the skin layer and elucidating the proteolytic properties of the papain enzyme in breaking down this deposition. However, further investigation is warranted to fully elucidate the primary causes of protein deposition and explore the diverse pathways involved in its accumulation within the cutaneous tissue. Moreover, there is a pressing need for more extensive research aimed at identifying novel sources of therapeutic agents, especially leveraging the rich natural resources

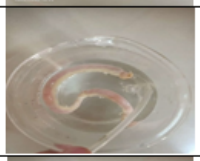
Images	Observation
	On the day 1 the patch was very minor as it is initial stage for protein to show its reactivity on the ileum. The patch was of 1,5 cm as shown in image
	On the second day the ileum started showing its reactivity. The patch of the protein got increased up to the 2 cm.
	Day 3 (Image c): On the day 3 the proteins patch was increased due to its growth stage. The patch was of 2.5 cm.
	Day 4 (imaged): On the fourth day the patch was observed to be increased by 0.5 cm that was 3cms. The images are attached for the reference.
	Day 5(image e) On the fifth day, the patch got increased up to 3.5 cm. The ileum was a bit swollen as clearly seen in the image. Hence it was confirmed that the overdose of protein or if the amount of protein is increased in the body it may cause a disease called macularamyloidosis.

Figure 8: In vitro observations:

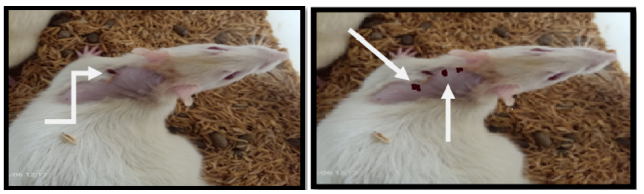


Figure 9: Induction of macular variant of cutaneous amyloidosis as black to brownish coloured spot

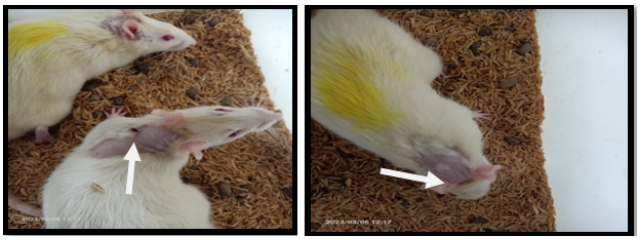


Figure 10: Recovery of macular variant of cutaneous amyloidosis which indicated as black to brownish coloured spot

abundant in the Indian subcontinent. By delving deeper into these areas of inquiry, we can advance our understanding of skin disorders and potentially uncover new avenues for the development of effective treatments.

4. Source of Funding

None.

5. Conflict of Interest

None.

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