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REVIEW

Production, purification, characterization, immobilization, and application of Serrapeptase: a review

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BACKGROUND: Serrapeptase is a proteolytic enzyme with many favorable biological properties like anti-inflammatory, analgesic, anti-bacterial, fibrinolytic properties and hence, is widely used in clinical practice for the treatment of many diseases. Although Serrapeptase is widely used, there are very few published papers and the information available about the enzyme is very meagre. Hence this review article compiles all the information about this important enzyme Serrapeptase.
METHODS: A literature search against various databases and search engines like PubMed, SpringerLink, Scopus etc. was performed.
RESULTS: We gathered and highlight all the published information regarding the molecular aspects, properties, sources, production, purification, detection, optimizing yield, immobilization, clinical studies, pharmacology, interaction studies, formulation, dosage and safety of the enzyme Serrapeptase.
CONCLUSION: Serrapeptase is used in many clinical studies against various diseases for its anti-inflammatory, fibrinolytic and analgesic effects. There is insufficient data regarding the safety of the enzyme as a health supplement. Data about the anti-atherosclerotic activity, safety, tolerability, efficacy and mechanism of action of the Serrapeptase are still required.

Keywords Serrapeptase, proteolytic enzyme, anti-inflammatory, fibrinolytic

Introduction

Enzymes and enzyme pathways are an integral part of the human body. Enzymes are proteins in nature and act as biocatalysts for all the chemical reactions and hence act as therapeutic agents for most of the metabolic disorders (Cech and Bass, 1986). Enzymes are highly specific and increase the rate of a chemical reaction by lowering the activation energy without any alterations to the enzyme (Aldridge, 2013). Until the late 19th century, enzymes were used in treating only a handful of disorders like gastrointestinal disorders and as a digestive aid but the potential use of enzymes in infections, cancer and other diverse diseases have slowly emerged (Sabu, 2003). Enzymes are classified into six classes by the Enzyme Commission namely oxidoreductases, transferase, ligase, lyases, isomerases and hydrolases based on the type of reaction they catalyze (Singh et al., 2016).

Microorganisms are the most favorable sources of enzymes in contrast to plant or animal origin due to ease of availability and remarkable growth rate stability, ease of modification and production (Singh et al., 2016). Gene manipulations and genetic engineering of microorganisms can be easily performed using recombinant DNA technology to increase the rate of enzyme production (Illanes et al., 2012). Microbial enzymes have widespread applications in food, pharmaceutical, textile, paper, leather, medical and other industries and their demand is rapidly increasing over other conventional methods due to its greater efficiency, high-quality products and eco-friendly nature (Jordon, 1929; Kamini et al., 1999; Gurung et al., 2013).

When abundant protein particles aggregate in the body, protein lumps are formed, that obstruct arteries and organs causing multiple organ failure, which results in fatalities. Due to excess protein accumulation in diseased conditions, an overwhelming stress is created, affecting the tissues and thus the body works slower than usual to keep up with the protein decomposition. Hence, these proteins have to be eliminated from the body. Proteolytic enzymes thus aid in degrading protein masses accumulated in the body (Jickling et al., 2010). Proteolytic enzymes represent one of the three largest classes of enzymes, the hydrolases that can catalyze the

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hydrolysis of peptide bonds in proteins and peptides (Bach et al., 2012; Fadl et al., 2013). Proteolytic enzymes accounts for about 60% of total sale in the worldwide market (Anil and Kashinath, 2013).

Serrapeptase (EC number 3.4.24.40) is an effective proteolytic enzyme belonging to serine protease family that first came into interest to the Japanese biochemist 25 years ago, since when it has been used widely in health care in Asian and European countries. Serrapeptase made its debut in the United States in 1977. Serrapeptase is isolated initially from the Enterobacteria *Serratia marcescens* strain E-15 found in the gut of the Japanese silkworm *Bombyx mori*. It is also called as Serratiopeptidase or Serratia-peptidase as a reason of its origin from *Serratia marcescens* (Anil and Kashinath, 2013). Serrapeptase has an affinity to the dead proteins in the end of silkworm threads and dissolves the proteins that make up the cocoon and it selectively dissolves the proteins involved in non-living tissues found in the cocoon and not the living tissue (Sellman, 2003). Serrapeptase does not affect healthy tissues in the body because the chemical structure of Serrapeptase inhibits attachment to proteins in healthy tissues (Robert, 2009). The production of Serrapeptase depends upon a secretory protein on the membrane of the host cell and it is secreted by the N-terminal signal peptide-independent pathway (Kaviyarasi et al., 2016).

Serrapeptase has been analyzed to have a high degree of substrate specificity (Miyata et al., 1970b; Aiyappa and Harris, 1976). It is an immunologically active enzyme and it is anti-oedemic, analgesic, anti-inflammatory, solubilizes non-living tissues such as mucous, plaques and blood clots hence it is named as fibrinolytic/thrombolytic enzymes since it has the ability to degrade insoluble proteins like fibrin and other mediators of inflammation (Klein and Kullich, 2000). Serrapeptase is taken as a supplement that can boost the cardiovascular system and greatly augment overall health (Robert, 2009). The serine family proteases play important roles in not only obtaining nutrients but also in pathogenesis (Miyagawa et al., 1991). Serrapeptase can affect mammalian cells by degrading various protease inhibitors in the immune system and can also be the main factor behind infections in human epithelial cells (Shanks et al., 2015).

Serrapeptase has a potential to cure and treat disorders like atherosclerosis, arthritis, bronchitis, carpal tunnel syndrome, fibrocystic breast disease, Crohn's disease, leg ulcers, traumatic swelling, fibromyalgia, breast engorgement, migraine, Alzheimer's disease, sinusitis, hepatitis, lung disorders, arthritis, diabetes, carotid artery blockage, thrombosis, uterine fibroids (Klein and Kullich, 2000). Serrapeptase may also be used as a remedy for women suffering from endometriosis. Serrapeptase is also considered a healing enzyme as it heals sprained muscles, leg ulcers, traumatic injuries, torn ligaments, post-operative inflammation, drains mucus, reduces elasticity and viscosity of nasal mucus. It also drains pooling of fluids in mastectomies and dissolves lumps in the breasts (Sellman, 2003). Serrapeptase acts as an amyloid dissociating agent with a potential to degrade insulin amyloids and hence can be considered a potential drug for different amyloid associated diseases (Metkar et al., 2016). Serrapeptase has been found to thin the extracellular matrix thereby reducing the incidence of cancer metastasis (Robert, 2009). Serrapeptase is called the "miracle enzyme" or "super enzyme" due to its wide range of applications and actions on the human body (Anil and Kashinath, 2013). Research is still in progress to find the applications of Serrapeptase in treating other chronic disorders.

Molecular aspects of Serrapeptase

The molecular weight of Serrapeptase ranges about 45 kDa – 60 kDa (Fig.1). It is a metalloprotease and contains three zinc atoms as ligands and one active site (Hamada et al., 1996; Bhagat et al., 2013). The presence of zinc atom is essential and also enhances the proteolytic activity of Serrapeptase. The structure of Serrapeptase as containing three zinc ligands was predicted and confirmed by comparing the structure of Serrapeptase with thermolysin and *Bacillus subtilis* neutral protease (Anil and Kashinath, 2013). The gene encoding Serrapeptase reveals that it is made up of 470 amino acids. The amino acid sequence is free of Sulfur containing amino acids, cysteine and methionine (Matsumoto et al., 1984). The G + C content of the coding region for the mature protein is 58% (Nakahama et al., 1986). The maximum enzyme activity of Serrapeptase is observed at pH 9.0 and at a temperature of 40°C. Serrapeptase is degraded or inactivated completely at a temperature of 55°C (Kaviyarasi et al., 2015).It possesses an isoelectric point of 5.3 (Matsumoto et al., 1984). It is an active enzyme that binds to the α-2 macroglobulin in biological fluids and in blood, it binds in the ratio of 1:1 and this binding helps mask its antigenicity, retaining the enzymatic activity (Anil and Kashinath, 2013; Juhi et al., 2015).

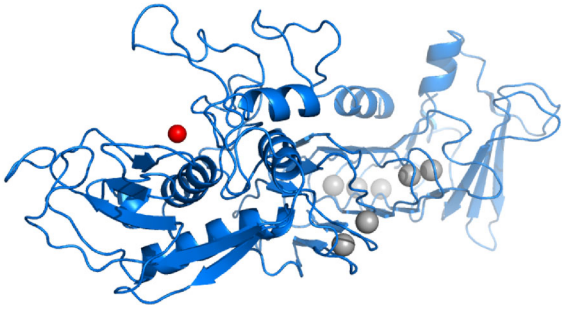

Figure 1 Crystal structure of Serrapeptase generated by PyMOL software.

The gene for Serrapeptase has been cloned and sequenced (Nakahama et al., 1986; Braunagel and Benedik, 1990) and

its crystal structure has been determined (Baumann, 1994). Docking studies reveal that inhibitors of Serrapeptase like EDTA and Lisinopril show favorable interaction and binding at the zinc binding site of Serrapeptase with minimal free energy (Kaviyarasi et al., 2016). Genetic characterization of *Serratia marcescens* can be done indirectly by 16s rRNA sequence analysis, neighbor joining method, phylogenetic analysis and peptide mass fingerprinting of Serrapeptase. Peptide mass fingerprinting is used to elucidate the amino acid sequence of Serrapeptase and can be confirmed by performing a pairwise alignment (Mohankumar and Raj, 2011). Homogenous purification for the large-scale production of Serrapeptase by conventional methods from the isolated organism is intricate but homogenous preparation is required for various applications and characterization. Thus, the versatile rDNA approach is ideal to achieve homogeneous commercial grade Serrapeptase (Kaviyarasi et al., 2015).

The Serrapeptase gene from *Serratia marcescens* E-15 was originally cloned into pTSP26 and expressed in *E. coli*-JM 103 but the expressed Serrapeptase was found inside the *E. coli* cells and not in the culture medium (Nakahama et al., 1986). In another study, observations showed that the Serrapeptase gene from *Serratia marcescens* strain SM6 expressed in *E.coli* using a lac promoter was secreted into the medium but as an inactive protein with a marginally higher molecular weight (Braunagel and Benedik, 1990). In addition, Serrapeptase gene from *Serratia marcescens* HR-3 was expressed in *E.coli (DE3)/pLysS* strain using the expression vector pET32a (+) and reported that the enzyme was highly expressed as inclusion bodies and the purified Serrapeptase was found to be dormant (Tao et al., 2007). Hence, we can conclude that the problem of secreting Serrapeptase into the medium using *E. coli* may be due to the fact that *Serratia marcescens* secretion genes are not being clustered near the Serrapeptase structural gene or due to the incorrect processing of the protease zymogen, which is dysfunctional in *E. coli* (Letoffe et al., 1991).

Recombinant Serrapeptase was produced by cloning Serrapeptase gene into a pET28b vector and expressing it in *E. coli* BL21. This resulted in the absence of inclusion bodies in the cytoplasm, with the recombinant proteins secreted properly in the extracellular medium despite a small amount of it in the intracellular sample (Selan et al., 2015). The Serrapeptase gene was also cloned into pJET 1.2 cloning vector and expressed in *E. coli* DH5-α and proper cloning of Serrapeptase was observed by sequencing. The sequence analysis reported the presence of a single Open reading frame (ORF) comprising of 1464 nucleotides. The sequence obtained showed 100% homology with serralysin metalloprotease from *Serratia marcescens* strain 2170 (Kaviyarasi et al., 2015). Hence pET28 and pJET series vectors are most suitable for expression of Serrapeptase in *E. coli* without forming inclusion bodies. Serrapeptase gene was also cloned into pPICZαA Pichia expression vector and electrotransformed into *Pichia pastoris* GS115 and maximum expression was found to be at 72 h Apart from *E. coli* expression system, Yeast can also be a favorable alternative host for the expression of proteins (Kaviyarasi et al., 2016).

Therapeutic properties of Serrapeptase

Anti-inflammatory

Chronic inflammation is an epidemic of the 21st century. Inappropriate diet, high glucose level, food intolerance, aging are certain factors influencing inflammation and pain (Sellman, 2003). All diseased conditions generate a certain amount of inflammation which is proportional to the aggressiveness of the disease. Inflammation provokes the immune system into exempting and activating the white blood cells that travel through the circulatory system annihilating pathogenic bacteria, foreign substances and cancer cells that it encounters. White blood cells can escape into organs and tissues during their repair mechanisms and this accelerated activity of the white blood cells results in tissue damage (Robert, 2009). Serrapeptase thus helps to abate the inflammation in arteries that promotes accumulation of cholesterol and narrowing of the arteries and hence is used to treat atherosclerosis, artery blocks and other cardiovascular diseases (Fig.2) (Liver doctor, 2013). Serrapeptase reduces inflammation in 3 ways: 1. by breaking down insoluble protein by-products like fibrin, 2. By thinning the fluids formed during injury which in turn speeds up tissue repair process, 3. Reducing pain by inhibiting the release of pain-inducing substances like amines (Sellman, 2003). It can also modify the cell adhesion molecules that are involved in guiding inflammatory cells to the site of infection (Klein and Kullich, 2000). It is orally effective in treating inflammation caused by laryngitis, catarrhal rhino-pharyngitis, sinusitis,

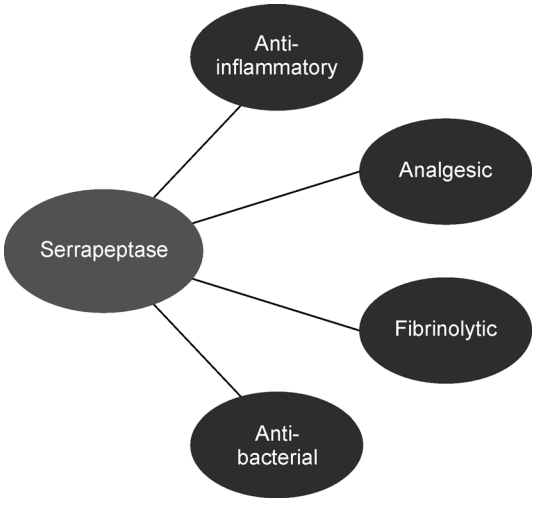

Figure 2 Properties of Serrapeptase.

breast engorgement, carpal tunnel syndrome, inflammation in prostate gland, acute and chronic ear-nose-throat disease, and chronic emphysema (Tachibana et al., 1984; Vicari et al., 2005; UmaMaheswari et al., 2016).

Analgesic

Serrapeptase reduces pain by restricting the inflamed tissues from releasing pain-inducing amines such as bradykinin (Mazzone et al., 1990). It can also hydrolyze bradykinin, histamine and serotonin, which are responsible for oedemic responses (Malshe, 2000). Docking studies have revealed that the substrate bradykinin that binds near the zinc binding site of Serrapeptase can be effectively inhibited by cleaving the peptide bonds of bradykinin (Kaviyarasi et al., 2016). It is used in treating various diseases as an alternative to salicylates, ibuprofen and other NSAIDs (Non-steroidal anti-inflammatory drugs) (Aso et al., 1981).

Fibrinolytic

Fibrin belongs to a category of proteins that are naturally adept in repairing damage occurring from trauma, surgery and injuries by replacing the old cells with new cells, tissues, and muscles (Jickling et al., 2010). When a tissue is damaged, the blood vessels secrete a compound called thromboplastin and simultaneously the platelets adhere to the broken blood vessels releasing platelet factor. Now both thromboplastin and platelet factor react with calcium ions and other factors to form prothrombin activator that is converted into insoluble fibrin which accumulates as a clot in the body obstructing the blood flow, oxygen supply to tissues, causing myocardial infarction, strokes in the brain, pulmonary emboli and thrombi in veins (Guyton, 1974). Hence dissolving the clot is necessary to avoid significant risk of damage to the body and this is where these fibrinolytic enzymes play an important role.

Serrapeptase has the ability to digest non-living tissues such as mucous, plaques and blood clots. Since it has the ability to degrade insoluble protein like fibrin without harming other living tissue, it is designated as a fibrinolytic enzyme (Klein and Kullich, 2000). Serrapeptase possesses the ability to dissolve and reduce arterial plaques, fatty cholesterol, calcium and other foreign protein substances from sticking to arterial walls. Serrapeptase is used as an alternative to chelation therapy as it is more effective than EDTA-mediated chelation for removing arterial plaques by dislodging the excess fatty deposits in the arteries allowing optimum blood flow which lowers the blood pressure and arterial resistance (Nieper, 2010). Serrapeptase, therefore, helps people with limited mobility such as those affected by orthostatic hypotension, which is characterized by a drop-in blood pressure brought about when a person changes their body position (Liver Doctor, 2013).

Anti-pathogenic agent

Bacteria create a biofilm within the microbiome beneath which they thrive. *Staphylococcus aureus* possesses a number of virulence factors and has the ability to invade eukaryotic cells and forms surface biofilms causing staphylococcal infections. Blocking *S. aureus* colonization may reduce the incidence of invasive infectious diseases. The anti-infective properties of Serrapeptase can be used in impairing staphylococcal properties like attachment to inert surfaces and invasion on eukaryotic cells. But the exact mechanism of action is yet to be elucidated (Selan et al., 2015). Thus, the anti-biofilm efficacy of Serrapeptase may enhance the antibacterial effects on staphylococcal infections. Serrapeptase has antibacterial activity on *Escherichia coli* and *Pseudomonas aeruginosa* with a zone of clearance of 15mm and 12mm respectively and the maximum antibacterial effect seen in dialysis based partial purification of Serrapeptase (Devi et al., 2013).

Sources of Serrapeptase

Serrapeptase is produced by a variety of microorganisms isolated from different sources (Table 1). Soil and contami-

Table 1 Bacterial strains that produce Serrapeptase

Sl. No.	Name of the bacteria	Strain	Source	References
1	*Serratia marcescens*	E 15	Intestine of silkworm	Anil et al., 2013
A	*Serratia marcescens*	VITSD2	Soil	Robert et al., 2009
3	*Serratia marcescens*	NRRL B-23112	Soil	Salamone et al., 1997
4	*Serratia* sp.	ZF03	Hot springs	Salarizadeh et al., 2014
5	*Serratia marcescens*	NCIM 2919	Not mentioned	Wagdarikar et al., 2015
6	*Bacillus licheniformis*	NCIM 2042	Not mentioned	Wagdarikar et al., 2015
7	*Serratia marcescens*	SRM	Flowers of summer squash	Kaviyarasi et al., 2016
8	*Streptomyces hydrogenans* var.	Not mentioned	Mangrove soil	Nageswara et al., 2016
9	*Streptomyces hydrogenans*	MGS1	Soil	Vanama et al., 2014
10	*Serratia marcescens*	P3	Soil	Bach et al., 2012

nated water are a rich source of a diverse variety of microorganisms. Isolated pure cultures of the bacterial strains that produce Serrapeptase are maintained on nutrient agar plates and stored at 4°C (Devi et al., 2013). Serrapeptase was first isolated from *Serratia marcescens* strain E-15 found in the gut of the silkworm *Bombyxmori* (Sellman, 2003).

Serratia marcescens is a Gram-negative bacterium that belongs to *Enterobacteriaceae* family that can grow in a wide range of temperatures (5–40°C) and pH (5.0–9.0) and secretes a variety of enzymes such as serine and thiol proteases, metalloproteases, lipases, chitinases, hemolysin, and nucleases (Jayaratne, 1996). *Serratia marcescens* can be differentiated from other Gram-negative bacteria by its ability to hydrolyze casein (Stancu, 2016). It is well known for producing a cell-associated, red pigment called prodigiosin, which resembles human blood. Factors such as medium composition and oxygen supply, affect the production of prodigiosin and incubation at 37°C inhibit the pigmentation which makes it tough to identify it in a pool of bacteria (Gerber, 1975). It is pathogenic to both humans and plants, involved in food spoilage (Abdou, 2003) and acts as a powerful insecticide (Salamone and Wodzinski, 1997). It promotes plant growth by inducing resistance against plant pathogens (Kloepper et al., 1993), producing antagonistic substances (Queiroz and Melo, 2006) and solubilizing phosphate molecules (Tripura et al., 2007).

Production of Serrapeptase

Strains producing Serrapeptase especially *Serratia marcescens* are usually cultured in trypticase soy broth (Fig.3). A medium containing carbon source- maltose, organic nitrogen source- peptone, inorganic nitrogen source- ammonium sulfate, dihydrogen phosphate, sodium bicarbonate, inorganic salt source- sodium acetate, glycerin and ascorbic acid can be used as a production medium and this medium yielded about 27.36 U/ml (Badhe et al., 2009). Another medium reported for production of Serrapeptase contained maltose 45 g/l, soybean meal 65 g/l, KH_2PO_4 8.0 g/l, and NaCl 5.0 g/l at a pH 7.0 which gave a maximum yield of 32575EU/mg. This maximum yield was due to presence of maltose as carbon source (Pansuriya and Singhal, 2010). A combination of tryptic soy broth (30 g/l) and skim milk (5% w/v) (TSB-SM) medium can also be used which is equal to Serrapeptase production using glucose minimal medium for 48 h with subsequent addition of 10% (w/v) skim milk at intervals of 12 h (Salamone and Wodzinski, 1997). Casein medium can also be used but trypticase soy is a preferred substrate over casein as the specific activity is higher when trypticase soy is used as the substrate in the production medium (Devi et al., 2013). Another medium containing tryptone, yeast extract, glycine, sodium chloride, skim milk 1% (w/v) and 0.5% (w/v) glucose is used for Serrapeptase production. Induction of the

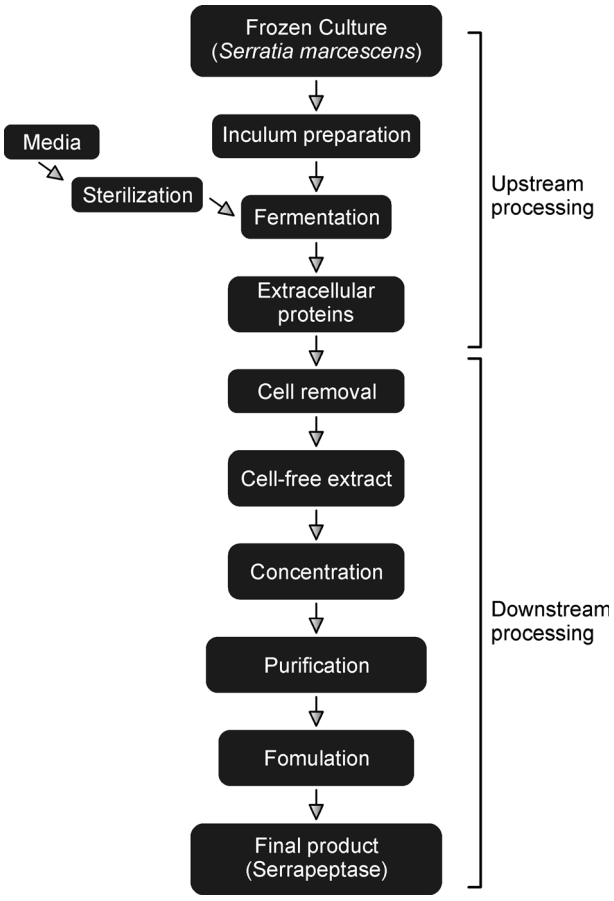

Figure 3 Industrial processing of Serrapeptase.

Serrapeptase enzyme was due to the presence of Skim milk and glucose (Romero et al., 2001). Feather meal broth can also be used for enzyme production that contains feather meal, sodium chloride, KH_2PO_4 and K_2HPO_4 (Bach et al., 2012). The enzyme produced can be filtered using filter paper and stored at 4°C for further use (Mohankumar and Raj, 2011).

Growth curve analysis of Serrapeptase shows that Serrapeptase production is observed at 12 h of growth time and maximum production can be observed at 48 h of growth time (Devi et al., 2013). The medium used for production of Serrapeptase by *Streptomyces hydrogenans* contains soya bean meal, glucose, glycerol, $CaCO_3$, tryptone and KH_2PO_4 (Vanama et al., 2014). Horse gram (*Microtylona uniflorum*) is one of a few low-cost substrates for the production of Serrapeptase by *Streptomyces hydrogenans* var. under solid state fermentation conditions (Nageswara et al., 2016). The enzyme produced is expressed in terms of units/ml using a special standard curve (Ammar et al., 1998; Mohankumar and Raj, 2011). The medium used for production of Serrapeptase by *Bacillus licheniformis* contains glycerin, glucose, tryptone, ammonium oxalate, sodium acetate, disodium hydrogen phosphate and ammonium sulfate at a composition

of 10 g/l each maintained at a pH of 7.5 to get a yield of 22.85 IU/ml (Wagdarikar et al., 2015). The growth medium used for production of Serrapeptase in yeast is Minimal medium with Histidine (MMH) and the production medium used is minimal medium with Glycerol and Histidine (MGYH) and a maximum yield of 0.6 mg/ml was obtained using these medium (Kaviyarasi et al., 2016).

Purification of Serrapeptase

Partial purification of the enzyme can be performed by ammonium sulfate precipitation, dialysis, ultra-filtration, aqueous two-phase systems, High-Performance Liquid Chromatography (HPLC) etc. (Bach et al., 2002; Devi et al., 2013). Serrapeptase can also be purified using ultrasound assisted three phase partitioning method, which not only purifies the enzyme but also concentrates it. This method has several advantages like single step purification, easy scale-up, economical and accounts for about 96% recovery of Serrapeptase with a 9.4-fold degree of purification in 5 min of process time under optimal conditions, 30% w/v ammonium sulfate concentration, 1:1 t-butanol to crude ratio, pH 7.0, 0.05 W/cm^2 ultrasonic intensity, 25 kHz frequency and 20% duty cycle (Pakhale and Bhagwat, 2016). The activity of Serrapeptase, estimated by gelatin clearing zone increased from 24mm zone of clearance to 36mm zone of clearance after purification of the enzyme by ammonium sulfate precipitation (Anil and Kashinath, 2013). Casein assay showed that specific activity of Serrapeptase in the crude enzyme, precipitated and dialyzed samples to be 12.00 U/ml, 21.33 U/ml, and 25.7 U/ml respectively, with a maximum purification fold of 2.1 in dialyzed samples followed by a 1.8-fold purification in precipitated samples and a 1-fold purification in the crude samples and hence proves that partial purification by ammonium sulfate precipitation and dialysis gives better enzyme activity than the crude enzyme (Devi et al., 2013). Another study showed a maximum purification fold of 5.7 for dialysis followed by 3.8 for acetone fractionation, 1.8 for ammonium sulfate precipitation and a 1-fold purification for crude samples (Salamone and Wodzinski, 1997). A 2013 study reported a maximum specific activity of ammonium sulfate precipitated Serrapeptase to be 63623 EU/mg and dialysis purified Serrapeptase to be 190451 EU/mg (Ayswarya et al., 2013). Recombinant Serrapeptase was purified in a single step using Nickel-NTA based affinity chromatography in which His_6 tag helps in purification and the yield of the purified Serrapeptase was found to be 0.6mg/ml (Kaviyarasi et al., 2016). Hence, we can conclude that dialysis is the best method for partial purification of Serrapeptase. Complete purification of the enzyme can be achieved by Chromatographic methods among which reverse phase HPLC is so far used. Reverse phase HPLC can be used for purification and is regarded as a versatile, accurate, robust and the precision values lay under the ICH guidelines of validation. This method resulted in a 100.23% - 100.71% recovery of the Serrapeptase (Patel et al., 2015). Another study of Reverse phase HPLC purification showed a percentage recovery of 98.9% to 99.5% (Reddy et al., 2015). Hence chromatographic techniques can be used for high rate of purification of enzymes.

Detection and determination of activity of Serrapeptase

Serrapeptase can be estimated by widely used methods such as reverse phase HPLC, UV based methods, radioimmunoassay, Lowry's assay (Lowry et al., 1951), Bradford assay etc (Miyata et al., 1970). Proteolytic activity of Serrapeptase can be detected by skim milk agar plate method where a clear zone formation indicates the enzyme has proteolytic activity (Salarizadeh et al., 2014). Proteolytic activity of Serrapeptase can also be distinguished by well diffusion method in which culture filtrates will be added in wells in the agar medium and stained and de-stained to visualize a clear zone around the well (Devi et al., 2013). Serrapeptase has absorption maxima at 275-280 nm (Anil and Kashinath, 2013). The enzyme activity of Serrapeptase can be determined by casein protease assay or by gelatin clearing zone assay (Salamone and Wodzinski, 1997). The enzyme's kinetic parameters, K_m and V_{max} values can be determined by Lineweaver-Burk plot according to Michaelis-Menten kinetics and were found to be 0.00105 mg/ml and 0.0531 mM/min, respectively (Salarizadeh et al., 2014). HPLC can also be used to detect the presence of Serrapeptase. The enzyme extract with a maximum retention time of 3.45 min was observed by this method (Devi et al., 2013). Reverse phase HPLC was used for estimation of enzyme and a correlation co-efficient of 0.998 was obtained in this study which is closely equal to 1 and hence suggests good concentration of purified enzyme (Patel et al., 2015). Another study evaluating the activity of Serrapeptase by Reverse phase HPLC showed a regression co-efficient of 0.998, limit of detection at a concentration of 3.33 μ/ml, limit of quantitation of 10.9 μ/ml (Reddy et al., 2015).When trypticase soy broth is used as the substrate, the maximum specific activity of Serrapeptase is found to be 60.7U/mg with a clearance zone of 23mm on a skim milk agar plate (Devi et al., 2013) but when casein is used as the substrate, specific activity of Serrapeptase is found to be 0.65U/ml (Subbaiya et al., 2011). Serrapeptase was estimated in formulations by using microplate readers which use the principle of vertical photometry. A linear relationship was observed between Serrapeptase concentration and absorbance at 230 nm with the co-efficient of regression being 0.9911, percentage recovery was found to be 97%- 98%, abiding the standard limits, low standard deviation of ± 0.020 to ± 0.044 which confirms the method to be precise, accurate and free from any positive or negative interference of the excipient (Sandhya et al., 2008). The presence of Serrapeptase was

detected using a zymogram which produced a clearance zone. The recombinant Serrapeptase had an enzyme activity of 30 U/ml and specific activity of 50 U/mg (Kaviyarasi et al., 2016).

Optimization studies on production of Serrapeptase

The media composition, temperature, pH, and other conditions can be optimized for increased yield of Serrapeptase with better enzymatic activity.

Effect of media composition

Maximum production of Serrapeptase can be obtained when both tryptone and yeast extract are added to the medium, in the absence of glucose is absent in the medium (Anil and Kashinath, 2013). The richest source of carbon is glucose for *Serratia marcescens*, both Glycerin and Maltose for *Bacillus licheniformis*. The best nitrogen source is tryptone for both *Serratia marcescens* and *Bacillus licheniformis*. After optimizing the media for *Bacillus licheniformis* the concentration of Serrapeptase increased from 16.52 IU/ml to 22.85 IU/ml (Wagdarikar et al., 2015). In optimizing batch process study, the production medium containing tryptone, together with maltose as carbon source gave a maximum activity of 36,415 EU/mg at the 68th hour (Ayswarya et al., 2013). The suggested amino acid for maximum yield of Serrapeptase is valine. The addition of any acids would lead to inhibition of Serrapeptase production (Mohankumar and Raj, 2011).

A surface response method of media components was considered to enhance Serrapeptase yield by *Streptomyces hydrogenans* MGS13. Response surface method is an empirical statistical modeling technique used for multiple regression analysis to solve multi-variable equations simultaneously. Here, the medium for maximum production was optimized by 'one-variable-at-a-time approach by studying effects of dextrose, soybean meal as substrate variables, pH, inoculum level. The coefficient of determination (R^2) was estimated to be 0.9559 for Serrapeptase production which is statistically significant as R^2 lies from 0 to 1 and 0.9559 is nearly equal to 1 which implies that the model is valid. Maximum Serrapeptase production of 254.65 IU/ml was observed with dextrose and soybean meal concentrations of 2.04 (%w/v) and 2.09 (%w/v) respectively (Vanama et al., 2014). Another study used 1% soybean meal as optimal nitrogen source to obtain a maximum yield of 85U/gds from *Streptomyces hydrogenans* (Nageswara et al., 2016). The Gelatin Clearing Zone (GCZ) exhibited by Serrapeptase produced from *Serratia marcescens* shows maximum Serrapeptase production with a clearing zone of 36mm at gelatin concentration of 0.5% w/v and higher or lower concentrations of gelatin results in decrease in Serrapeptase production (Mohankumar and Raj, 2011). 5 g of horse gram is the optimal substrate concentration for maximum Serrapeptase yield of 85 U/gds from *Streptomyces hydrogenans* (Nageswara et al., 2016).

Effect of agitation and aeration

Aeration and agitation rates are both key parameters in the fermentative production of Serrapeptase. The maximum specific productivity, 78.8 EU/g maltose/hour, has been obtained at the optimum fermentation conditions of 400 rpm agitation and 0.075 vvm aeration with the maximum yield of 11580 EU/ ml (Ruchir et al., 2011) which is the highest yield to date (Decedue et al., 1979; Miyazaki et al., 1990).

Effect of temperature

Temperatures between 32°C (Mohankumar and Raj, 2011) and 37°C (Anil and Kashinath, 2013) show maximum Serrapeptase production from *Serratia marcescens*. Hence 32°C – 37°C is generally the favorable temperature for maximum Serrapeptase production. Temperatures below or above this range decreases the yield (Anil and Kashinath, 2013). The optimum temperature for maximum Serrapeptase production from *Bacillus licheniformis* is about 35°C (Wagdarikar et al., 2015). Serrapeptase is stable up to 42°C and activity of the enzyme rapidly decreases above 42°C (Salamone and Wodzinski, 1997). Serrapeptase is optimally active in the range of 50°C-55°C, and at 45°C it retained 85% of its enzyme activity and declined to 25% at 60°C (Salarizadeh et al., 2014). In a comparative study of exposing *Serratia marcescens* to different temperatures of 28°C, 32°C, 37°C, and 40°C, it was observed that activity of enzyme retained till a maximum temperature of 32°C (Manal, 2015).

Effect of pH

Optimum pH for maximum Serrapeptase production from *Serratia marcescens* is 5.0 to 9.0 with phosphate buffer as the best buffer and a notable decline in productivity can be seen at both higher and lower pH values from the optimum pH value (Mohankumar and Raj, 2011). Even at pH 9.0, the enzyme is in its active form and is stable (Salarizadeh et al., 2014). Another study found that the optimum pH for maximum Serrapeptase production from *Serratia marcescens* was 7.3 (Anil and Kashinath, 2013). Optimum pH for maximum Serrapeptase production from *Bacillus licheniformis* was 6.5 (Wagdarikar et al., 2015). Optimal pH for maximum Serrapeptase yield of 85 U/gds from *Streptomyces hydrogenans* was found to be 6.5-7.0 (Nageswara et al., 2016).

Effect of incubation period

Incubation time is very important in determining the yield and activity of any enzyme. The optimum incubation period for Serrapeptase production from *Serratia marcescens* varies

from 24 h (Mohankumar and Raj, 2011) to 25 h (Anil and Kashinath, 2013). The optimal incubation duration for maximal Serrapeptase production from *Bacillus Licheniformis* is 24 h (Wagdarikar et al., 2015).

Effect of mutations

Mutations can be induced by different means and the easiest way is by exposure to UV light. In a study, *Serratia marcescens* isolates were exposed to UV light for different time intervals of 20, 40, 60seconds and was observed that the maximum hydrolysis of casein was at 20 s of UV exposure at 32°C (Manal, 2015). Another study confirmed that the maximum Serrapeptase activity of 1575.3 EU/ml was observed at 20 s of UV exposure (Ayswarya et al., 2013). *Streptomyces* also are regarded as an efficient producer of Serrapeptase. *Streptomyces* isolates were subjected to nitrous acid treated chemical mutation and observed a maximum activity of 60.1% higher than wild-type strain. When the same strain was subjected to UV mutation with a UV lamp of 220 V, 40 W, 50 Hz with the exposure time of 0, 30, 60, 90, 120, 150, 180, 240 and 360 s, it was observed that UV mutant exhibited 33.9% higher activity than the wild-type strain (Vanama et al., 2014).

Immobilization of Serrapeptase

Immobilization of drugs and other biological agents these days are used for targeted drug delivery. Immobilized enzymes have several advantages like the fact that they can be recycled easily, removed easily from the reaction mixtures, minimal amount of enzyme is lost in the reaction mixture, have greater thermal stability (Yu et al., 2012). The idea of immobilization emerged from the advent of nanotechnology.

Magnetic nanoparticles are widely used for immobilization of enzymes. Magnetic nanoparticles have several advantages over other materials including the fact that they are easy to prepare, come in a wide range of sizes, chemically modifiable, superparamagnetic in nature, possess large surface area, low mass transfer, highly active, inert,and highly stable (Kumar et al., 2010; Krukemeyer et al., 2012; Verma et al., 2013).

MNPs can be removed easily from the body through a process called opsonization (Chen et al., 2011). Some magnetic nanoparticles (MNPs) used for immobilization of Serrapeptase are Fe_3O_4 nanoparticles, carboxyl-functionalized magnetic nanoparticles, Amino-functionalized magnetic nanoparticles (Namdeo and Bajpai, 2009), chitosan/glutaraldehyde MNPs, 3-amino propyltriethoxysilane (APTES) magnetic nanoparticles etc. (Kumar et al., 2014). Other materials used for enzyme immobilization include Gold, ionic fluids, albumin, streptavidin, polymer-coating cellulose, dextran, silica (Bi et al., 2009; Ziv-Polat et al., 2010; Yu et al., 2012). Alginate based microspheres encapsulated with Serrapeptase have also proved to be very efficient in wound healing therapies (Rath et al., 2010).

When Serrapeptase was immobilized using ethyl cellulose microparticles, the entrapment efficiency was found to be 85% and the yield, calculated using the weight of the raw materials and the microparticles obtained was found to be 96%. Serrapeptase undergoes metabolism causing gastrointestinal disturbance and systemic toxicity which can be overcome by using a transdermal drug delivery system. In this system, lipid-based transferosomes are used to delivery Serrapeptase with greater encapsulation of about 90% due to the presence of more cholesterol. It showed a greater tensile strength of 2.95 ± 0.71 to 2.98 ± 0.89 kg/cm^2 and hence showed no risk of cracking.The drug release was slow and controlled when compared to percentage release from an aqueous solution of Serrapeptase (Pravin et al., 2015). When Serrapeptase was immobilized with carboxyl- functionalized magnetic nanoparticles, the kinetic parameters K_m increased from 0.096 mg/ml to 0.121 mg/ml, V_{max} decreased from 0.061 to 0.045 µmol/min. Serrapeptase immobilized on carboxyl functionalized magnetic nanoparticles was found to be better than amino-functionalized magnetic nanoparticles with a yield of 115.78 mg protein/g (Kumar et al., 2013). When Serrapeptase was immobilized on Fe_3O_4 magnetic nanoparticles, the kinetic parameter, Km of free and immobilized enzyme was found to be 0.078mg/ml and 0.1 mg/ml respectively showing a significant increase in Km of Serrapeptase after immobilization due to factors like change in structure of enzyme, steric hindrance, effects of diffusion etc., (Zhu et al., 2009). *In vivo* studies suggest that magnetic targeting has the potential to increase the permeation of Serrapeptase through the membrane and also enhance the anti-inflammatory effects in carrageenan induced paw edema in rats even at very less dose ofimmobilized enzyme than required. V_{max} for immobilized enzyme also decreased from 0.064 µmol/min to 0.055 µmol/min respectively and relative activity for immobilized enzyme was found to be 67.875. Studies have also found that immobilization of Serrapeptase does not affect the crystallinity and has very less effect on the size and magnetic properties of nanoparticles used (Kumar et al., 2013).

Pharmacokinetic studies

Serrapeptase is usually administered orally. Serrapeptase is considered a systemic enzyme in humans as they are absorbed by the intestines and then is directly released into the bloodstream (Moriya et al., 1994). Absorption studies on proteolytic enzymes have confirmed that they are absorbed intact into the bloodstream in its active form and are actively transported through the gut wall (Ambrus et al., 1967). Serrapeptase, when used therapeutically may lead to low bioavailability due to low membrane permeability and enzymatic degradation (Woodley, 1994). At an oral dose of

100 mg/kg, Serrapeptase can be detected in the serum and lymph of rats at a concentration of 0.87 +/ − 0.41ng/mL and 43 +/ − 42ng/mL respectively. This was measured by an enzyme immunoassay technique with a maximum time interval of 15-30 min.This study reveals that Serrapeptase is absorbed by the intestinal tract and transferred to the inflammatory site via blood or lymph in an enzymatically active state (Moriya et al., 1994). To improve the oral absorption, a liposomal formulation of Serrapeptase can be used as liposomal mediated delivery shows a 50% increase in Serrapeptase entrapment efficiency but the permeation value in terms of a log was found to be −7.72cm/s which is lower than the Biopharmaceutics Classification System (BCS) classification (Ruell et al., 2002; Sandhya et al., 2008).

Clinical studies of Serrapeptase

The potential effects of Serrapeptase in different disease conditions have been tested on both animals and humans (Fig.4, Tables 2 and 3).

Contraindications

Since Serrapeptase is very effective at degrading fibrin, there may be increased bleeding when taken with natural agents like turmeric, garlic, certain oils like fish oil and chemical agents like Aspirin and Warfarin. Serrapeptase cross reacts with statins, NSAIDs, anticoagulants, anti-platelet agents, heparin and heparinoids (Bhagat et al., 2013).

Formulation of Serrapeptase

Serrapeptase, being an enzyme, can be degraded easily by other digestive enzymes in the stomach and gastrointestinal tract. Therefore, Serrapeptase is manufactured as enteric coated tablets. The enteric coating is basically a polymer

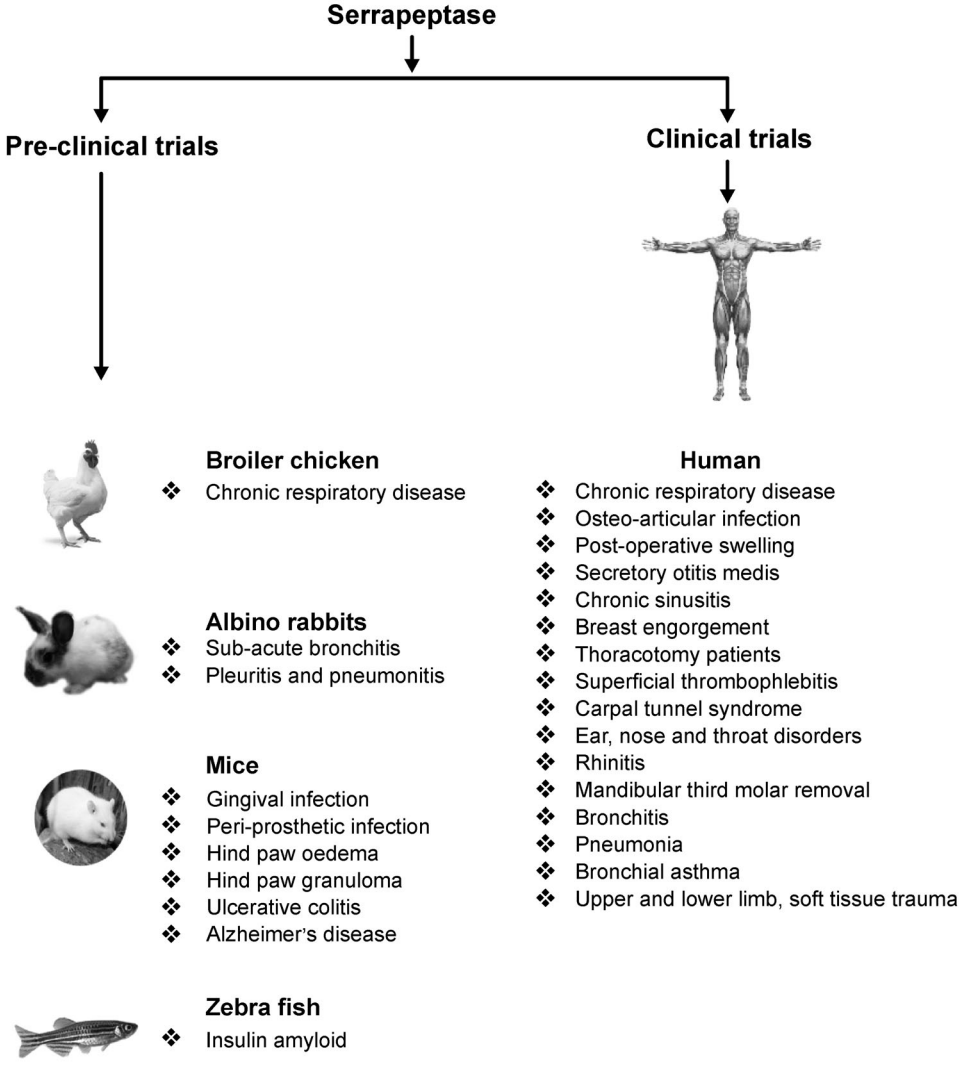

Figure 4 Pre-clinical and clinical studies of Serrapeptase.

Table 2 Pre-Clinical studies

Sl. no	Disorder	Dosage	Time(d)	Study animal	Outcome	Reference
1	Gingival infection	STP (20mg/kg) + 4 antimicrobial (100mg/kg orally)	Not mentioned	Rat	Serrapeptase enhanced the activity and concentration of antimicrobials used	Aratani et al., 1980
	Sub-acute bronchitis	20mg/kg	Not mentioned	Rabbit	Reduced viscosity of sputum	Kase et al., 1982
	Pleuritis and pneumonitis	STP + Cefotiam	30min after injection	Rabbit	Serrapeptase increases plasma concentration of cefotiam	Ishihara et al., 1983
	Peri-prosthetic infection	STP + antibiotic	14 days	Rat	Serrapeptase eradicated infection caused by biofilm- forming bacteria and increased antibiotic efficacy. In 5.6% cases, the infection persisted	Mecikoglu et al., 2006
	Hind paw oedema and granuloma	STP	3 hours	Albino rats	Serrapeptase showed enhanced anti-inflammatory activity	Viswanatha et al., 2008
	paw oedema	3mg/kg	3 hours	Rats	Serrapeptase reduced inflammation and reduced oedema by 44%	Mundhava et al., 2016
	Chronic paw oedema	20mg/kg	8 hours	Albino rats	Serrapeptase significantly inhibited inflammation induced by formalin by 68%	Jadav et al., 2010
	Insulin amyloid	0.55mg/ml	2-32 hours	Zebrafish	Serrapeptase degraded the amyloid fibrils formed by insulin	Metkar et al., 2016
10	ulcerative colitis	1.3mg/kg	Not mentioned	Albino female mice	Serrapeptase showed significant anti-inflammatory activity	Rajinikanth et al., 2014
11	Chronic respiratory disease	2 g/litre	3days	Broiler chicks	Serrapeptase significantly decreased the total cholesterol, serum LDH and the inflammatory markers CRP and ESR, better immunological response to NDV vaccination and decreased the shedding of Mycoplasma gallisepticum and *E. coli*	El-Hamid et al., 2014
12	Alzheimer's Disease	10.800 U/ kg b.wt and 21.600 U/ kg b. wt	45 days	Albino rats	Histopathological investigation of Alzheimer's induced rat brain tissue showed the disappearance of most of the amyloid plaques after Serrapeptase treatment and also decreased cholinesterase activity, TGF- β, IL-6 and P53 levels accompanied with a significant increase in Bcl-2 level	Ahmed et al., 2014
13	Alzheimer's disease	17 mg/kg b.wt	45 days	Albino rats	Serrapeptase decreased brain AchE activity, TGF-b, Fas and IL-6 levels and increased the expression levels of ADAM9 and ADAM10 genes in the brain tissue of the treated rats, which are factors of Alzheimer's disease	Fadl et al., 2013

Table 3 Clinical Studies of Serrapeptase

Sl.no.	Disorder	Dosage	Time (days)	No. of patient	Study design	Outcome	Reference
1	Osteo-articular infection	STP 30mg/d + sulbenicillin	6	8	Open	Reduced the inflammation by transferring sulbenicillin to cell exudates	Okumura et al., 1977
2	Chronic respiratory disease	10mg, thrice daily	14	376 (128 on STP)	Random, double-blind	No significant improvement	Nagaoka et al., 1979
3	Post-operative buccal swelling	30mg/d	7	174 (88 on STP)	Random, double-blind	Significant reduction in swelling of the buccal cavity	Tachibana et al., 1984
4	Secretory otitis media	0.5mg/d	10	75	Random, double-blind	Tests unclear	Bellussi 1984
5	Chronic respiratory disease	30mg/d	7	40	Random	Relaxation of sputum elasticity was observed	Shimura et al., 1983
6	Chronic sinusitis	30mg/d	30	Not mentioned	Open	Reduction in the viscosity of mucus was seen	Majima et al., 1988
7	Breast engorgement	30mg/d	3	70	Random, double-blind	Improvement in breast pain, swelling	Kee et al., 1989
8	Lung cancer patients undergoing thoracotomy	20mg Thrice daily	Not mentioned	35 (18 on STP)	Random, open	STP group patients had more amounts of antibiotics in their tissues	Koyama et al., 1986
9	Post-operative swelling	5mg thrice daily	5	40	Not given	No significant improvement	Surachai et al., 1981
10	Post-operative and traumatic swelling	Not given	Not clear	66	Random	The pain disappeared on the 10^{th} day of treatment and swelling reduced by 50%	Esch et al., 1989
11	Post-operative and traumatic swelling	Not given	Not clear	98	Random, double-blind	Reduced swelling	Tsuyama et al., 1977
12	Superficial thrombo-phlebitis	30mg/d	14	40	Random	65% patients taking STP showed a reduction in pain and improvement in other symptoms like erythema	Bracale et al., 1996
13	Carpal tunnel syndrome	10mg twice daily	44	20	Prospective trial	65% of patients showed improvement in symptoms	Panagariya et al., 1999
14	Acute and chronic ear, nose, and throat disorder	30mg/d	7-8	193	Random, double-blind	Reduced pain, amount of secretions, difficulty in swallowing and nasal obstruction	Mazzone et al., 1990
15	Chronic airway disease	30mg/d	30	29 (15 on STP)	Random, open	Reduced solid component, viscosity, the elasticity of sputum and sputum neutrophil count	Nakamura et al., 2003
16	Perennial rhinitis, chronic rhinitis	STP + Cephalexin	Not mentioned	93	Random, double-blind	rhinorrhea, nasal stuffiness were reduced due to STP intake	Brewer science., 1999
17	Removal of mandibular third molars	5mg STP + 1000mg paracetamol	7	24	Individual, random, double-blind	Reduction in cheek swelling and pain	Khateeb et al., 2008
18	Post-operative swelling after removal of molars	5mg thrice daily	5	40	Not given	No improvements in symptoms	Chopra et al., 2009
19	Bronchitis, pneumonia, bronchial Asthma	30mg/d	14	140 (69 on STP)	Random, double-blind.	Serrapeptase was found to be effective and safe expectorant in controlling the sticky sputum formation	Kai-sheng et al., 2009
20	Upper and lower limb, soft tissue trauma	30mg/d	14	100 (50 on STP)	Prospective trail	Serratiopeptidase showed a significant anti-inflammatory effect and mild analgesic effect	Garg et al., 2012

which is sensitive to pH i.e., these tablets remain intact in acidic pH of the stomach and the gastrointestinal tract and gets dissolved in the alkaline pH of the small intestine (Bodhankar et al., 2011). Liposomal formulations of Serrapeptase are used as effective oral drug delivery systems as it has increased permeability and hence can increase oral absorption of Serrapeptase (Sandhya et al., 2008). Lipid-based transferosomes are also one mode of enzymatic carriers of drugs that are studied recently (Pravin et al., 2015).

Dosage of Serrapeptase

Serrapeptase doses usually ranges from 10 mg to 60 mg per day. Most pharmaceutical firms formulate the drug dosage to be 10mg, taken 2-3 times a day. 10mg corresponds to about 20000 enzyme units of Serrapeptase. Serrapeptase must be taken on an empty stomach. Also, the person should not consume any food up to half an hour after taking Serrapeptase (Bhagat et al., 2013). If it is consumed with a meal, then the body will utilize it to digest the food (Liver Doctor, 2013). It is to be ensured that Serrapeptase should be double dosed.

Safety of Serrapeptase

There are limited adverse drug reactions reported so far for Serrapeptase. They include skin conditions like dermatosis, dermatitis, erythema, muscle and joint aches, coagulation abnormalities (Mazzone et al., 1990). There may also be certain gastric related issues like nausea, anorexia, stomach upset, cough, pneumonitis (Sasaki et al., 2000). Serrapeptase may also cause granulomatous hepatitis (only 1 case reported so far), acute eosinophilic pneumonia (Dohmen et al., 1998; Sasaki et al., 2000). Serrapeptase may induce hemorrhage and hence while consuming this drug or any proteolytic drugs to prevent thrombosis, bleeding risk should be taken into consideration (Celik et al., 2013). It has no inhibitory effects on prostaglandins and is free from serious effects like stomach ulceration, joint destruction, kidney problems, stomach upset, psychiatric problems (Sellman, 200). A 69-year-old man developed acute renal failure following treatment with diclofenac/Serrapeptase which also led to leg swelling and tests revealed pedal edema with concentration of urea 99 mg/dL, plasma creatinine 9.4 mg/dL, plasma sodium 133 mEq/L and plasma potassium 4.9 mEq/l, reduced urinary sodium and osmolality and hence Serrapeptase was withdrawn (Dhanvijay et al., 2013).

Conclusion and future perspectives

Enzymes from microbial sources play a vital role in pharma and health care. Serrapeptase is being used in many clinical studies against various diseases for its anti-inflammatory, fibrinolytic, anti-bacterial and analgesic effects. It is also recommended for preventing cardiovascular blocks due to its fibrinolytic activity. Although many bacteria produce Serrapeptase, *Serratia marcescens* is the best producer of Serrapeptase. Many studies on production strategies, purification, optimization studies and immobilization of the enzyme have been studied and reported to be successful and efficient. Preclinical studies on various animal models and clinical studies have shown significant effects for various diseases. Absorption study confirms that the Serrapeptase is absorbed through the intestines and released into the bloodstream. Serrapeptase may have side effects if taken along with anti-fibrinolytic agents and hence care must to take when consuming Serrapeptase. Serrapeptase is manufactured as enteric coated tablets to avoid getting degraded by other digestive enzymes. When Serrapeptase is used to treat any disorder, it should never be consumed along with meals, as it will be utilized to digest food. Data regarding the safety and drug interaction studies of the enzyme is insufficient to use it as a health supplement. Data and research about the anti-atherosclerotic activity, safety, tolerability, efficacy and mechanism of action of the Serrapeptase are still required to be widely accepted for treating various diseases.

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Compliance with ethics guidelines

E. Selvarajan and Shreya Gopinath declare that they have no conflicts of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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