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Development of thermostable Streptokinase by recombinant DNA technology as fibrinolytic agent for different thromboembolic disorders

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ABSTRACT

Background: Streptokinase is presently utilized in clinical medicine as a therapeutic agent in the management of thromboembolic blockade. Streptokinase is an extracellular protein secreted by certain strains of beta-hemolytic streptococci. It activates plasminogen to produce plasmin, an enzyme that breaks down fibrin clots through specific lysine binding sites. **Purpose of research:** Production of thermostable streptokinase by recombinant DNA technology as a fibrinolytic agent for various thromboembolic diseases. The aim of this study was to improve the physicochemical properties of the streptokinase enzyme to increase its thermal stability and exhibit superior fibrinolytic activity against various thromboembolic diseases. **Research type:** Screening experimental study. Our study type was to screen the thermostability and biological activity of modified streptokinase generated by bioinformatics. **Methodology:** Isolation of SPP-producing streptokinase was performed on blood agar. Streptococcus agalactiae was the main isolate in screening studies. In this work, streptokinase was generated by bioinformatic recombinant DNA techniques by placing cysteine-cysteine side by side in the core alpha-helix of this fibrinotyl enzyme. The streptokinase product was pre-extracted by salt precipitation with ammonium sulfate and then purified by affinity chromatography. A casein digestion method was used to detect and determine the biological activity of test enzymes compared to standard streptokinase enzymes. The expression host was Pichia pastoris SMD1168. The C-terminus is 6x histidine, the inducer is methanol, the promoter is AUG1, and PYES2-DEST52 is an expression vector. **Result:** This research led to improvements in the physicochemical properties of the streptokinase enzyme, making it a thermostable drug. **Conclusion:**Thermostable streptokinase protein produced by recombinant DNA technology, showed high efficacy as a thrombolytic agent for dissolution of various throboembolov disorders.

Introduction

Thromboembolic difficulties are taken into consideration a main motive of dying worldwide. Streptokinase is a fibrinolytic agent [1] It is used withinside the dissolution of clot [2] Fibrinolysis refers back to the technique of fibrin digestion with the aid of using the fibrin-particular protease, plasmin [3] The fibrinolytic device is much like the coagulation device in that the precursor shape of the serine protease plasmin circulates in an inactive

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shape as plasminogen [4]. In reaction to injury, endothelial cells synthesize and launch tissue plasminogen activator (t-PA), which converts plasminogen to plasmin[5] Plasmin remodels the thrombus and bounds its extension with the aid of using proteolytic digestion of fibrin[6]. Streptokinase is a protein (however now no longer an enzyme in itself) synthesized with the aid of using streptococci that mixes with the proactivator plasminogen[7].This enzymatic complicated catalyzes the conversion of inactive plasminogen to lively plasmin[8] Urokinase is a human enzyme synthesized with the aid of exploiting the kidney[9] that immediately converts plasminogen to lively plasmin[10]. Plasmin itself can not be victimized due to the fact evidently taking place inhibitors in plasma save you its consequence[11].Nonetheless; the absence of inhibitors for urokinase and the streptokinase-proactivator complicated allow their employment clinically[12]. Plasmin shaped inner a thrombus with the aid of victimizing those activators is blanketed from plasma antiplasmins,[13]which permits it to lyse the thrombus from within[14].

Disadvantages of streptokinase protein encompass Bleeding, thermolability[15]. This investigation aimed to conquer the legal responsibility downside the use of new drug shipping structures containing changed thermostable streptokinase designed with the aid of exploiting genetic engineering and peptidomimetics and display notable fibrinolytic avocation for exclusive thromboembolic troubles.

Materials and Methods

Ethical statement:

In the present study, we followed All applicable national, international, and/or institutional guidelines for the attention and utilization of humans and animals. All processes carried out in the study including humans and animals were authorized by the local authorities, the Ethical committee for human and animal handling at Cairo university(ECAHCU), at the Faculty of Pharmacy, Cairo University, Egypt in agreement with the recommendations of the Weatherall report with approval number P-8-1-2021. All efforts were performed to ablate the number of humans and animals utilized and their suffering during the study.

Inclusion criteria for animal models are : Adult animals; can be induced by thrmboembolic disorder such as rabbits and mice; obese animals. Exclusion criteria are: Young animals; Pregnant female animals; Non-obese animals. The type of study: Screening experimental study. Adult obese male rabbits weighing about 2kg, and obese male albino mice were utilized in the existing study. Mice were acclimatized for one week before the experiment. At a humidity(50%±5), light-dark cycle (12/12 h), and a controlled temperature (25±2 0C). Mice were provided with a commercially accessible natural diet of chow(Elnasr pharmaceutical and chemical company).

Material and equipment: All chemical and biochemical material were purchased from Algomhoria pharmaceutical company, Cairo, Egypt and Alnasr pharmaceutical company, Abo zabal Alkhanka,Qalyobia,Egypt.

Source of animal models: They were obtained and legalized from pharmacology and toxicology department of faculty of pharmacy,Cairo university, Egypt. Place and date of the study: This study was done in microbiology and immunology department in faculty of pharmacy Cairo university and thoracic and cardiology surgery department of faculty of medicine, Cairo university, Egypt. It was finished between January 2021 and April 2022.

Methods

Design of primer for expression of recombinant thermostable stable streptokinase:

Forward primer: GCAACCGGGTGGAATATTGC

Reverse primer: GCTGCAGAACTCTGAGCTGT

Improvement of the conformation and physical stability of protein with high therapeutic value:

Isolation, screening and identification of streptokinase producing bacterial species were performed by exploiting fibrinogen selective media followed by subculturing the positive isolates on blood agar selective media from 50 different throat swabs. Furthermore; Separation of the gene of the therapeutic protein of interest was carried out using proper type II endonuclease enzymes(Hind III and Xor II), succeeded by insertion of it inside a suitable host for its expression such as E.coli using PUC18 expression vector plasmid and ligase enzyme. Moreover; Purification of the enzyme of interest from the supernatant of centrifuge tube as

extracellular protein was achieved by crystallization by ammonium sulfate. Characterization of the optimal physiological and environmental conditions of enzyme production and activity were performed via biochemical reactions. Screening of therapeutic effect and determination of optimal dose for biological activity was finished through animal models. Determination, and formulation of a suitable dosage form and route of administration such as intramuscular or intravenous injections were carried out later [16]. As well; the most flexible regions of the gene of streptokinase were detected by mistreatmenting conformational entropy and bioinformatics genetic engineering application software. This was followed by addition of disulfide bond via site directed mutagenesis in the form of induction of a mutation by a single nucleotide polymorphism utilizing pymol and DBD2 software in the flexible region of alpha helices of the core of the protein to substitute two residues of flexible amino acids such as glycine with two cysteine residues adjacent to each other and are not apart by more than 0.2 nm from each other to form intermolecular and intramolecular disulfide bonds which increase the physical stability and the conformation of the protein of interest. Mutants were generated by 60 0C gamma irradiation of a Streptococcus agalactiae culture of stationary cells in LB medium, under continuous flushing with oxygen. Insertion of the mutated gene of protein of interest was performed in a suitable expression host such as Pichia pastoris SMD1168(purchased from Stratagene, USA)which secreted the protein of interest extracellulary. C-terminal was 6x histidine, inducer was methanol, promotor was AUG1 and PYES2-DEST52 was expression system vector. As well genomic (cDNA) of streptokinase protein was extracted via victimization with restriction endonucleases type II such as Hind III and Xor II; moreover, was amplified by polymerase chain reaction(PCR) technique and subcloned to a prokaryotic expression vector PET 32a(obtained from Novagene, Germany), E.coli BL21(DE3) polysS((received from Novagene, Germany) were transformed with PET 32a and the gene expression was induced by IPTG. After a centrifuge for 3 minutes, the modified and mutated extracellular therapeutic protein was partially purified, extracted and precipitated from the supernatant of centrifuge tube by crystallization by ammonium sulfate, then completely refined aside from affinity chromatography technique through Ni-NTA resin. The yield of the recombinant streptokinase was assessed via gel electrophoresis method then confirmed with western blot technique, furthermore the molecular mass of streptokinase was determined by a mass spectrometer. E. coli DH5α(obtained from Stratagene, USA) was utilized as the primary host for the construction and the propagation of plasmids. LB agar and broth were in use for routine bacterial culture and the incubation time was for 24 hours at 37 C and PH 7.4. The antibiotics were added to the media according to to the references recommendations. The growth media for yeast growth were CSM media (purchased form creative-bioformat company, USA), they were exploited for achieving agar plates in order to grow Pichia pastoris SMD1168 competent cells.Moreover, YPD broth yeast media were utilized for maintenance and propagation of Pichia pastoris SMD1168 yeast strains [17] .Screening of physical stability and identification of optimal biological activity of the modified and mutated therapeutic protein were finished through animal models and tissue culturing testing [18].

Composition of fibrinogen selective agar medium:

Potassium chloride 0.5 mg, Magensium sulfate 0.5 mg, KH2po4 1.0 g, Ferrous sulfate 0.1 g, Zinc sulfate 0.1 g, Fibrinogen 1.0 g, Agar 2%.

Composition of blood agar medium:

Peptone 10.0 g/L, Tryptose 10.0 g/L, Sodium chloride 5.0 g/L, Agar 15.0 g/L, Distilled water 960 ml.

Final PH at 25 0C :7.3. The ingredients were combined then 5% sheep blood was added after autoclaving at 121 c for 15 minutes and before pouring onto the plates.

All ingredients were bought from Algomhuria company for chemicals in Cairo, Egypt.

Determination of Isolation, screening and identification of enzyme producing bacterial species using selective media:

Isolation of streptokinase producing SPP were done on a blood agar medium. Streptococcus agalactiae was found to be the main isolate producing Streptokinase protein from different throat swab samples in our screening study.The test

enzyme was precipitated from the supernatant of the test centrifuge tube by ammonium sulfate, then separated by affinity chromatography.

Method of the preparation of thermostable recombinant streptokinase:

Lyophphilized powder of recombinant streptokinase was obtained for preparation of a solution for injection 750000 units and 1500000 units in vials. The preparation was adminstered intravenously in the initial dose of 250000 IU in 50 ml of isotonic solution of sodium chloride during 30 minutes(30 drops per minute). Later, the adminstration of streptokinase in the dose of 100000 IU per hour was followed.

Evaluation tests of thermostable recombinant streptokinase produced from Streptococcus agalatiae:

These tests were carried out as per British pharmacopeia 2019

specifications.

Compatibility study: We characterized recombinant streptokinase and different excipients utilized in the preparation of intravenous and intraarterial injection formulations by FT-IR(Perkin-Elmer 1600 FTIR spectrophotometer) spectroscopy and DSC(Shimadzu-DSC 50) to see the compatibility. The optimized formulation was blended with 200 mg KBr; then compressed into discs which were scanned at 5mm/sec with a resolution of 1 cm-1 at a range of 4000-200 cm-1. Experiments of thermal analysis were carried out utilizing various scanning calorimeters (DSC). We heated the samples of the optimized formulation in hermetically sealed Aluminium pans at a temperature range of 0-4000 0C at a constant rate of 110 0C/minute under a purge of nitrogen(35 ml/min).

Hardness: We performed a diametric compression test according to British pharmacopeial technique 2.9.8 utilizing Monsanto hardness tester(USA). A hardness of 2kg/cm2 was acceptable in the case of intravenous or intraarterial drug delivery systems according to standard literature. For 20 powder sample we measured the pressure required to break a diametrically placed matrix powder, by a coiled spring.

Friability: We dedusted, accurately weighed, and placed a random sample(20 powder samples) of the whole samples corresponding to 6.5 g in the drum of a Roche friability tester. we rotated the drum 100 times and powder samples were accurately weighed, dedusted, and removed. 1% was considered acceptable as a maximum weight loss. In the Roche friability test apparatus, 20 powder samples were weighed and put in. The powder samples were uncovered to the recurrent shocks and rolling consequent of the falls inside the apparatus. The powder samples were dedusted after 100 processes. The percentage loss in the weight of the powders of streptokinase was the determining factor of the friability.

Wetting time: Two layers of rectangular absorbent paper (10cm×7.5 cm)fitted into a petri dish and wetted thoroughly with distilled water; were used for carrying out the test for wetting time. Then we placed the powder sample at the center of the plastic dish and recorded the time required for the water to diffuse from the absorbent paper using a stopwatch.

Disintegration test: The test was carried out according to British pharmacopeia 2019 standards. The type of disintegration time tester was DTGi made in Copley, England. We placed one powder sample of streptokinase in each of the six tubes and utilized distilled water maintained at 370 C; then powders were observed for disintegration. The basket from the fluid was lifted and observed for the powders' complete disintegration at the end of the time limit. DT

Weight variation determination: From each batch, 20 powders of streptokinase were chosen randomly and their average weights were calculated utilizing a digital weighing balance(Mettler Toledo, Switzerland); then percentage weight difference was estimated and checked with British pharmacopeia 2019 specifications.

Determination of water absorption ratio: We kept a piece of tissue paper folded twice in a petri dish (internal diameter 6 cm)incorporating 7 ml of purified water. Then we settled the powder samples on the tissue paper and left them to wet wholly. The wetted powders were separated and reweighed.

Determination of uniformity of drug content: From each formulation twenty samples of streptokinase were weighed and powdered (samples were placed inside a bottle then the cap was put back on and was turned clockwise until the samples were completely crushed and powdered); then10mg of the powder was weighed and dissolved in 100 ml of distilled water. we sonicated the mixture for 170 seconds and filtered it through Whatman filter paper No. 40. Then the filtrate was diluted with distilled water and the absorbance at 405 nm was estimated due to disulfide photolysis and covalent streptokinase tyrosine dimerization induced by UV light exposure.

In vitro drug release profile : Distilled water was used as the dissolution medium(300 ml) at 37 0C, PH 7.4, and 50 rpm(paddle) in presence of phosphate buffer 6.8. We collected samples(25ml) at 3,6,8,11,16,19,60,120,240 minutes intervals according to European pharmacopeia specifications 2020 and the withdrawn volumes were replaced by equivalent amounts of the plain dissolution medium. The amount of streptokinase released was measured using a UV spectrophotometer at 405 nm owing to disulfide photolysis and covalent insulin tyrosine dimerization induced by UV light exposure. The type of dissolution tester was DISi made in Copley, England.

Stability study: It was carried out for optimized formulation. The storage conditions utilized for stability studies were accelerated conditions at 40 0C and room temperature at 30 0C. Optimized formulation powders were kept, striped, and packed in a humidity chamber for thirty days at above mention temperature. The parameters that were measured before and after the storage for one month comprised hardness, percentage friability, disintegration time, and drug content.

Determination and detection of the biological activity of streptokinase:

Casein digestion method: Streptokinase activity was indirectly determined using the casein digestion method, which is based on the determination of tyrosine released from digested casein after plasminogen activation. Activity was determined absolutely victimizing the modified method from Sutar et al (1986). Reaction mixture (3 ml) incorporated 10 mg casein, 50 mM Tris-HCl, pH 9.0, 0.1 ml (or appropriate diluent) supernatant. Reactions were carried out at 37° C for 20 minutes and then stopped by the addition of 3.5 ml of 6% w/v trichloroacetic acid (TCA) and 0.3 ml of 4.4 M HCl. The reaction was then unbroken on ice for 30 minutes before filtering exploiting Whatman #1 filter paper. The absorbance of the TCA soluble fraction was deliberated at 280 nm. Units of enzymatic activity were measured utilizing thecurve of standard SK .

Fibrin clot digestion method: Streptokinase activates the conversion of plasminogen to plasmin, causing substrate hydrolysis (fibrin clot). The degree of hydrolysis was directly proportional to the concentration of the therapeutic enzyme of interest. The extent of hydrolysis was assessed by estimating the diameter of the thrombolysis zone on fibrinogen agar selective plates.

Chromozym activity test: Streptokinase activity was determined by a colorimetric method using N-ptosyl-glycyl-prolyl-lysine-p-nitroanilide acetate as substrate for the plasmin enzyme. Samples were mixed with plasminogen and the mixture was incubated at 37°C for approximately 6 minutes. A substrate mixture containing chromozyme dissolved in 50 mM Tris-HCl buffer, pH 9.0 was then added to the enzyme-substrate mixture. Reactions were incubated at 37°C for 20 minutes and absorbance changes at 405 nm were monitored at 37°C using a spectrophotometer. Units of enzymatic activity were calculated using a standard streptokinase curve.

Protamine sensitive electrode electrochemical method: The therapeutic enzyme of interest was electrochemically tested to determine its biological activity and optimal dose as a fibrinolytic agent. Three separate DNNS-based protamine-sensitive membrane electrodes were used simultaneously to monitor the initial decline in protamine levels. Experiments were performed by adding 5 microliters of 5 mg/mL protamine solution to 1 mL of Tris working buffer (50 mM Tris and 130 mM NaCl, pH 7.4). After reaching a steady-state/non-equilibrium response (3 min), a centrifuge of bacterial broth (40 µl), preincubation of 90 microliters sample (6 min) with human plasminogen (0.50 U, Sigma) and Tris - work buffer were performed. Decreases in EMF responses to protamine were monitored by each sensor for 6 min. A streptokinase standard curve was generated by plotting the initial rate of voltage drop in mV/min against the streptokinase action in the IU/mL sample.

Experimental venous thrombosis Technique:

Biological activity of the test streptokinase enzyme was compared to standard activity in a rabbit model of experimental venous thrombosis.

300 rabbit models were collected and divided into three groups (test, standard, and control). Each group consisted of 100 of rabbit models. In test and control groups, thrombosis was induced by jugular vein stasis and injection of 10 mg/kg thromboplastin into the ear vein of each rabbit model. Animals were randomized to receive IV test streptokinase 0.3, 1.0, 3.0, or 10.0 mg/kg or vehicle control and compared to standard intravenous streptokinase and vehicle control.

Human evaluation of different streptokinase drug delivery systems via randomized human clinical trials phases 1/2: 3 groups of adult thrmboembolic patients(such as pulmonary embolism with hemodynamic instability, acute myocardial infarction, deep venous thrombosis and ascending thrombophlebitis were included in our study. Each group consisted of 100 subjects:

Group(1)(negative control group) was managed with graded amounts of the placebo(100,000-250,000U/kg) by intravenous and intraarterial route of adminstration. Group(2)(positive control group) was administrated graded amounts of the standard standarad streptokinase(100,000-250,000U/kg) intravenously and intraarterially.

Group(3)(test group) was administrated graded amounts of the test recombinant streptokinase(100,000-250,000U/kg). The activity of streptokinase was estimated by the reduction in thrombotic disorder.

The diagnosis of thromboembolic disorder such as myocardial infarction with ST segment elevation was done clinically and confirmed by electrocardiography.

In vivo bio-availability study: Before dosing IV and IR injections 0.7-0.9ml of samples were withdrawn, and immediately after dosing at 30,60,120,240 minutes.Blood samples were further refrigerated and centrifuged at 4 0C within one hour of sampling. Insulin concentrations were determined using HPLC. HPLC analysis was through a reversed phase column utilizing phosphate buffer(PH 4.4) and acetonitrile(660/340, v/v) as mobile phase with a flow rate 0.9ml/min. The limit of UV estimation of streptokinase concentration in blood was at 405 nm. Area under the curve(AUC) and the % of relative bio-availability were measured. % of relative bio-availability was determined by the following equation:

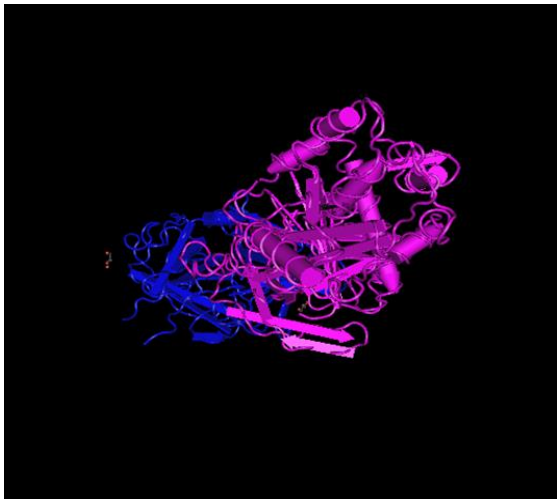
% Relative bio-availability=(AUC IV or IR/AUC Intravenous)×(Dose Intravenous/Dose IV or IR)×100%.

The same procedures were performed for the control and the standard groups(groups 1 and 2).

Determination of therm-stability of both the test and standard streptokinase proteins:

Both enzymes were stored at room temperature outside the refrigerator for one month.

Figure 1. It displays predicted 3D structure of recombinant thermostable streptokinase[3]**.**



[3]Streptokinase consists of 440 aminoacids.

Figure 2. It shows that about 86% streptokinase was purified by SDS gel electrophoresis and northen blot technique.

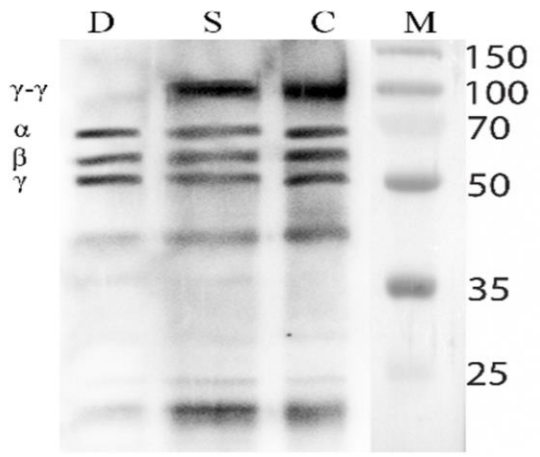


Figure 3. FTIR shows absence of any incompatibility or drug-drug interaction between recombinant streptokinase and excipients.

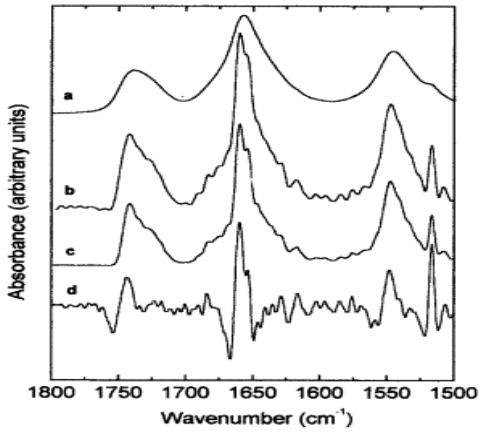
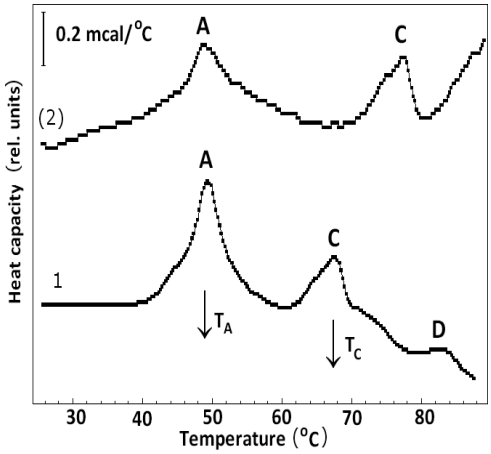


Figure 4. DSC shows absence of any incompatibility or drug-drug interaction between recombinant streptokinase and excipients.



Statistical analysis

All cultures were conducted in triplets. Their presentation was by means and standard deviation.One way analysis of variance (p value≤.05) was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software.

Figure 5. It displays the percentage of fibrinolysis of streptokinase during randomized human clinical trials stages 1/2.

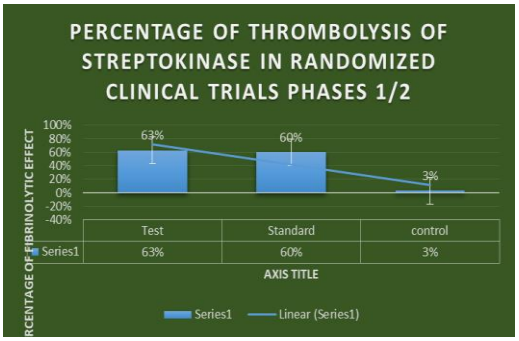


Figure 6. It displays the thrombolytic action of test recombinant thermostable streptokinase.

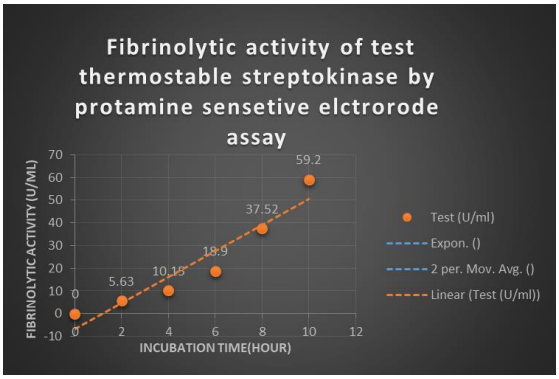


Figure 7. It displays the thrombolytic action of standard recombinant thermostable streptokinase

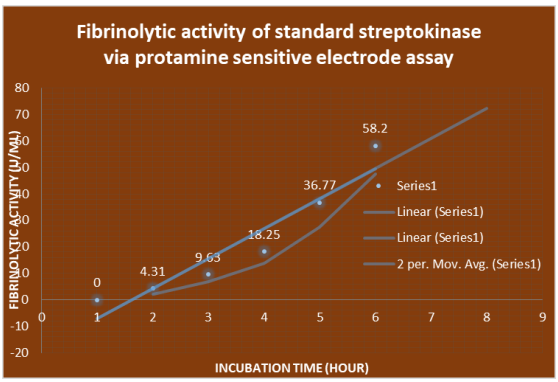


Figure 8. It represents fibrinolytic activity of test thermostable streptokinase by chromozym substrate assay.

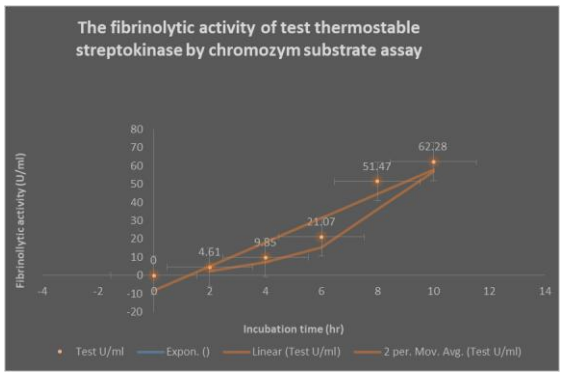


Figure 9. It represents fibrinolytic activity of standard thermostable streptokinase by chromozym substrate assay.

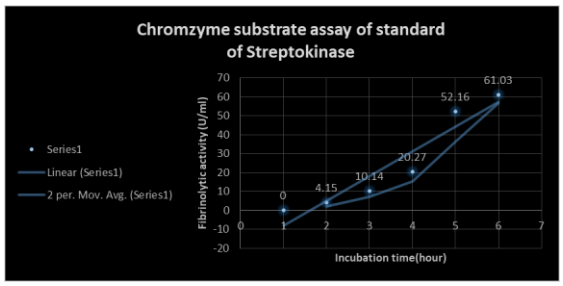


Figure 10. It represents AUC of recombinant streptokinase.

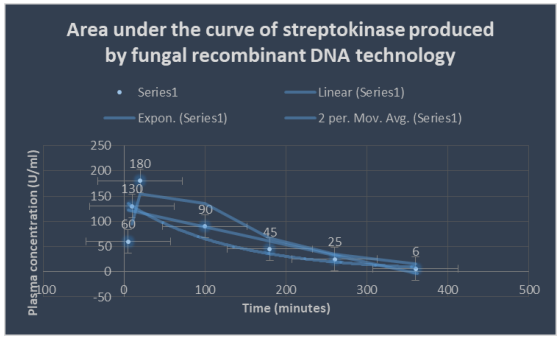


Figure 11. It represents fibrinolytic activity of test thermostable streptokinase by casein digestion assay.

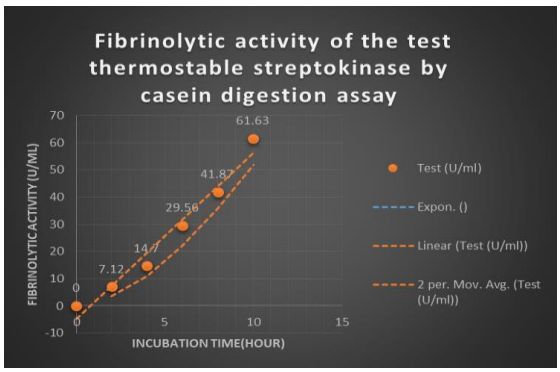
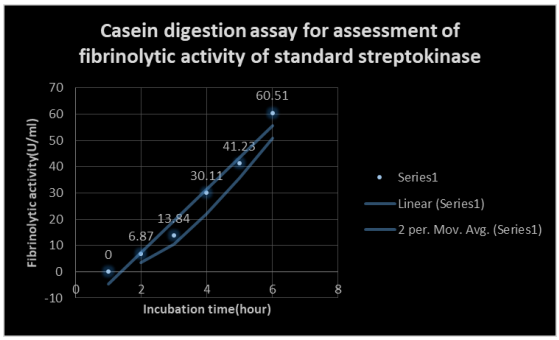


Figure 12. It represents fibrinolytic activity of standard thermostable streptokinase by casein digestion assay.



Results

The streptokinase enzyme's physicochemical characteristics were improved as a result of this research, making it a thermostable medication. In cases of thromboembolic stroke, pulmonary thrombosis, and cardiac thrombosis, the test enzyme has shown to be an effective fibrinolytic agent. Additionally, it was thermally stable enough to be kept at ambient temperature (2-8°C) rather than in the refrigerator. The common enzyme cannot be kept at room temperature since it is a heat-labile medication. The yield of recombinant streptokinase from the initial culture was about 470 mg/L. Different formulations(F1-F5) of recombinant streptokinase were prepared as shown in table 12. Formulation parameters were characterized as demonstrated in tables 3-6 and table 13. The pharmcokinetic profile of streptokinase was assessed. The biological half life of streptokinase was ascertained to be about 80 minutes and the duration of action was found to be 4 hours. Its fibrinolytic action was detected to be immediate. The peak plasma concentration was noticed to be 180 U/ml and was reached after 20 minutes.

Table 3 Batch formulation F1-F5 hardness, thickness, percentage Fri-ability, diameter and weight variation:

Batch	Hardness(kg/cm2)	%Fri-ability	Diameter(mm)	Thickness(mm)	weight variation(mg)
F1	1.99±0.35	0.57±0.02	6.02±0.01	3.3±0.02	130.21±1.1
F2	1.81±0.35	0.59±0.04	6.07±0.02	3.5±0.01	129.64±1.3
F3	1.74±0.36	0.63±0.01	6.04±0.01	3.4±0.04	132.53±1.4
F4	1.79±0.39	0.71±0.03	6.05±0.06	3.5±0.03	131.19±1.2
F5	1.88±0.46	0.68±0.01	6.06±0.03	3.6±0.07	132.28±1.7

Table 4. Drug content uniformity, wetting time, water absorption ratio, disintegration time of batch formulation F1-F5:

Batch	Drug content uniformity	Wetting time(sec)	Water absorption ratio	Disintegration time(sec)
F1	99.15 ±2.24	21 ±2.80	38.51 ±1.78	56 ±1.68
F2	97.78 ±1.36	25±1.91	40.14 ±2.19	57 ±2.71
F3	97.24 ±0.99	17 ±2.99	42.86 ±2.82	49 ±2.10
F4	98.77 ±1.78	23 ±1.87	38.47 ±1.42	45 ±2.08
F5	98.61 ±2.07	24 ±2.04	41.29 ±1.60	55 ±2.54

Table 6. Comparison of different parameters for stability study of batch F4 between its initial production and after the storage for one month :

Evaluation parameter	Initial	After one month
Drug content	98.77± 1.78	99.00 ±1.24
Hardness	1.79 ±0.39	1.83 ±0.38
Disintegration time(sec)	45.00±2.08	49.00 ±2.37
Percentage friability	0.71 ±0.03	0.74 ±0.02

Discussion

The present study type aimed to screen the therm-stability and the biological activity of streptokinase prepared by fungal recombinant DNA technology. The test fibrinolytic enzyme was indicated in animal candidates presenting within 24 hours of the onset of chest discomfort who had at least 1 mm of ST-segment elevation in two or more contiguous ECG leads. It opened approximately 76% of occluded blood vessels compared with less than 60% opened with the current streptokinase; while in randomized clinical trials it showed 63% efficacy. The test enzyme was extracellular protein. Its molecular weight was 47 KDa as determined by using a mass spectrometer. The yield of recombinant streptokinase was approximately 470 mg /L of initial culture. The test enzyme was thermostable and could be stored at room temperature outside the refrigerator(2-8 C). It showed approximately 76% efficacy as a fibrinolytic agent against occluded blood vessels while the standard one showed nearly 60%. The biological activity of the test enzyme was increased in presence of magnesium sulfate and thiamine chloride co-factors while it decreased in presence of ferrous sulfate cofactor. Determination of the biological activity of test enzyme was performed in comparison to standard streptokinase protein. The techniques utilized were Casein digestion method, Fibrin clot digestion method and Chromozyme substrate assay. Their Principals relied on that both test and standard streptokinase activated plasminogen to plasmin, the enzyme that degraded fibrin clots through its specific lysine binding site. Moreover, the extent of the digestion was directly proportional to the concentration of both enzymes. Protamine sensitive electrode electrochemical method principal was based on that the therapeutic protein of interest was assayed electorochemically for measurement of its biological activity and its optimal dose as fibrinolytic agent; then was compared with a standard streptokinase protein. The biological activity of test protein was found to be directly proportional to the concentration as compared with standard protein of streptokinase.The test protein exhibited improved a fibrinolytic activity. The biological activity of test streptokinase enzyme was compared with standard one on rabbit models of experimental venous thrombosis. The test thermostable streptokinase showed 76% fibrinolytic activity, while the standard one showed only 60% fibrinolytic activity against venous thrombosis during animal testing preclinical trials. In clinical trials phases 1/2, it showed 63% efficacy while it was less than 60% for current standard one[19].

Determination of therm-stability of both the test and standard streptokinase proteins.

The test therapeutic protein was found to be thermostable and could be stored at the room temperature outside the refrigerator while, the standard protein was found to be thermolabile and could not be stored at room temperature outside the refrigerator[20].

No possibility of interaction between recombinant streptokinase and excipients was shown by FT-IR and DSC studies[21]. The determination of the hardness of the powders of streptokinase for injection was done and was observed between 1.74 to 1.99 kg/cm2. The variation of weight of all formulations was estimated which were within the standard limit as per British pharmacopeia 2019 specifications. We found percentage friability in the range of 0.57 to 0.71% which was within the limit of extent. The ratio of water absorption for all formulations was observed between 38.47 to 42.86. The wetting time for all formulations was estimated between 17 to 24 seconds. We subjected the streptokinase powders for evaluation of in vitro disintegration time. For formulations F1 to F5, in vitro disintegration time was found to be in the range of 45 to 55 seconds. A rapid disintegration time of 45 seconds was observed by formulation F4. This was because of the burst effect and the rapid water uptake from the medium[23]. All formulation's percentage drug content was observed between 97.24% to 98.77% of recombinant streptokinase which was to an unexceptionable extent. The release time for the streptokinase powder intended for intravenous and /or intraarterial injection ranged from 97.81% to 99.26% in 15-18 minutes at 37 C and 50 rpm. Batch F4 displayed quicker drug release than all the other batches. 98.26 % cumulative drug release in 15 minutes was demonstrated by batch F4. Batch F4 biological half-life(t50 %) of the immediate release streptokinase powder was observed to be 4 minutes; while it was 3 minutes for standard streptokinase powder[22]. Owing to the rapid disintegration time and dissolution profile Batch F4 was well-advised as an optimized formulation. The optimum storage temperature of recombinant streptokinase(batch F1 to F5) was noticed at 250C. We performed in vivo study by taking formulation F4 and the outcome was compared with intravenous stanadrd streptokinase. At different time intervals, the blood samples were withdrawn, then analyzed for the drug content utilizing HPLC.

Tmax and Cmax of recombinant streptokinase were determined to be 20 minutes and 180 U /ml at an average dose of 100000 U/kg. % relative bio-availability was estimated by equation 1 and was dictated to be approxinately 93%. Bio-availability has been improved by recombinant streptokinase as was incontestable by results of in vivo study. T max of intravenous injection of streptokinase was 21 minutes and C max was approximately 175 U/ml at an average dose of 100000 U/kg. The onset of action of IVstandard streptokinase was about10 minutes, its duration of action was approximately 5 hours and its bio-availability was about 90%.

Limitation of study:

This included the small sample size and unavailability of enough resources.

Conclusion

By using bioinformatics,our screening investigation may be able to enhance the physicochemical properties of the existing fibrinolytic streptokinase protein. In our work, the modified protein displayed good efficacy and thermal stability. It is advised to switch to our novel enzyme in place of the existing streptokinase because it shown greater bioavailability, thermal stability, and efficacy. In comparison to the present streptokinase, which opened less than 60% of blocked blood arteries, the test enzyme opened about 63% of them in human clinical trials phases 1/2.

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