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A Review Article: Protein Engineering of Therapeutic Enzymes

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ABSTRACT

Through the development of advanced, stimulus-responsive pharmacological systems, protein engineering has the potential to alter the metabolic drug landscapes. Protein therapies are a fast growing category of FDA-approved medications that have the potential to improve clinical consequences in the long run. Protein therapeutics engineering is still in its preliminary phase; however recent advancements in protein engineering skills are being used to gain direct monitoring over pharmacodynamics. Drugs that are intended to be metabolized under specific conditions are known as stimulus-responsive protein medicines. Protein engineering is being utilized to develop biochemically based smart medicines that are tailored to the specific needs of each patient's condition. To meet the requirements of therapeutic applications, protein engineering adds new features and functionality to the process of bio-chemical and bio-physical modification of proteins. Given the immense potential of protein engineering approaches to adjust the activity of bio-catalysts, this review reviews the current studies that demonstrate both the advancements and the limitations of using these methods to therapeutic enzyme engineering in the pharmaceutical industry. The current review will concentrate mainly on three types of therapeutic based enzymes: Diagnostic enzymes, Fibrinolytic enzymes, and pharmaceutical enzymes, in which the protein is the restorative agent, prodrug-activating enzymes, provokes a therapeutic effect, and diagnostic enzymes, in which the remarkable specificity and selectivity of a protein offer advantages compared to conventional analytical techniques.

Keywords- enzymes, stimulus-responsive, FDA, personalized.

I. INTRODUCTION

Enzymes now play a major role in the pharmaceutical industry, Because of their ability to quickly and selectively catalyse biochemical events in biological systems. Enzyme-based therapeutics is constantly becoming a research focus of current research, owing to their significant effects on human health. Enzymes are also able to comply with contemporary safety, healthcare, and environmental regulations as greener catalysts [1]. However, despite all of these exceptional characteristics, enzyme specificity increases costs. For instance, during the surface chemistry of interest, the binding between both the active site of an enzyme and a preferred industrial substrate still may not result in its transformation [2]. Relatively low operating catalytic efficiency and stabilization, on the other hand, is a barrier in enzymatic engineering settings [3]. As a result, intensive attempts are being made to modify enzymes to fulfil the demands of industrial applications. Enzyme engineering is a framework for modifying enzymes to achieve desired physicochemical and biological properties [4]. For decades, the production of therapeutic medicines for human disease has been a focal point of research and innovative expansion projects. Between 2004 and 2013, it is projected that $38 billion was spent on research projects to create new medication prospects [5]. Enzymes as therapeutics have a wide spectrum of applications in prescription drugs. Enzymes have a high sensitivity and specificity as well as selectivity for their particular substrates, which can reduce toxicity. Enzymes' catalytic capacities also enable them to transform several target molecules into desirable products, facilitating for the administration of lesser amounts of the treatment. These characteristics of enzymes make them particularly effective therapies, enabling them to do functions that conventional medications cannot while minimizing the negative effects associated with a lack of selectivity [6]. Because of the diverse array of enzymatic functions and therapeutic activities, there has been a lot of investigation into their potential clinical implications. Therapeutic enzymes pose new challenges since they are extracellular proteins that are injected into a human host to catalyse a clinically beneficial reaction. Such functionality has its own set of design criteria that are distinct from those used in protein and drug development. On either side, these enzymes can indeed be tailored to achieve degrees of selectivity and specificity that standard small-molecule medicines competitors cannot match. They must, on the other hand, avoid interfering with natural cellular activities, stay effective under physiological and pathological conditions (– for example, blood plasma), have appropriate profiles of pharmacokinetic, and provoke no smallest immunomodulatory reaction from the victim. The complexity and difficulty of developing therapeutic enzymes is significantly increased by these additional restrictions. For these reasons, considerable efforts have been made over last couple of decades to uncover novel biocatalysts for clinical applications that much overwhelm foregoing collection of enzymes that have received medical authorization. Moreover, PEGylation,

encapsulation in microparticles and nano-carriers, and modification of amino acid sequences in the main enzyme's active site to create variants with better therapeutic efficacy, half-life, and/or stabilization have also been investigated. Engineered enzyme medicines have been engineered to have improved distribution to target areas and fewer negative side effects (such as immunogenicity) when used continuously. Immobilization as well as protein engineering can be used to engineer enzymes [7]. Immobilization is the process of attaching enzymes to a solid framework that gives them structural stability. The improved stability of enzyme is due to this structural stiffness [8]. Protein engineering comprises the development of new enzymes or changes to the sequence/structure of present enzymes to fulfil desired functionality. Computational methods for protein engineering have become viable due to improvements in structural bioinformatics, biochemical force fields, and the accessibility of massive amounts of information about enzyme structure [9]. In protein engineering, an enzyme's structure is changed in order to change its characteristics [10] In this review, we look at the most recent advances in therapeutic enzymes, with a particular focus on protein engineering research. Discuss a number of enzymes that have been genetically engineered to expand their bio catalytic applications.

Therapeutic enzymes

Enzymes are used as medicinal agents in a variety of ways. However, despite their therapeutic properties, they are limited in their application due to a number of drawbacks such as instability under physiological environment, low affinity for substrates, predisposition for proteolytic cleavage, and supplementary catalytic activity, all of which cause adverse effects during diagnosis. All of these constraints are overcome via enzyme engineering, which turns an enzyme into a viable therapeutic target. Pharmaceutical enzymes are a subclass that includes proteins that make up the medicinal agent. Nucleases, Proteases, and esterases, along with enzymes that comprised if amino acids are descriptions. These enzymes' therapeutic efficacy is often boosted by improved catalytic performance and pharmacokinetics, along with lower immunogenicity. While increasing activity and stability is very simple and is frequently tackled by gene manipulation strategies [11, 12]. The following are some of the therapeutic enzymes that have been designed for high functionality.

Protein Engineering

Protein engineering is now possible due to the availability of long-acting and inhibitor-resistant thrombolytics. Protein engineering is the process of creating novel enzymes or proteins that have new or desirable capabilities. It is a relatively recent field of study. Based on the use of recombinant DNA technology, it is a method for altering amino acid sequences in proteins. The rapid growth of biological sciences, particularly recombinant DNA technology and eukaryotic research, has resulted in a wide range of protein engineering methodologies being made available to researchers today. Fibrinolytic catalysts aren't designed to increase half-life. One goal is to improve fibrin selectivity and hence lessen adverse effects [13, 14] or inhibiting receptor associations [15-17] Novel applications for pharmacological treatment, including the draining of intracerebral haemorrhages or the enlargement of the time frame in which thrombolytic can be delivered, are being developed as a result of reducing adverse effects [18, 19]. Despite advances in a number of essential features, such as half life, inhibitor resistance and fibrin selectivity, quicker thrombosis enzymatic activity remains a primary consideration. The structure and function correlations of fibrin-targeted medications must be investigated in conjunction with fibrin, which is a big molecule in and of it. Even massive multi-protein combinations will be solved due to advancements in structural techniques like Cryo-EM and lesser angle X-ray scattering technique. Different structural bioinformatics and computer simulations tools can be used to examine the acquired structural data, providing insights into the engineering activity, binding, specificity, and selection of troponins [19].

Protein Engineered Ribonucleases

Ribonucleases had been studied as anticancer therapies for more than 60- years [20].The discriminating ingestion of exogenous-RNases with subsequent interruption of RNA-metabolism is hypothesised to be caused by distinctions in features of surface between normal and cancerous cells. The cytotoxic characteristics of the enzymes are mediated by fast RNA breakdown in specific cells, which halts cellular proliferation and leads to mortality. Ranpirnase known as Onconase, an unaltered enzymes from the RNAse family. A subfamily identified from the Rana pippins frog, has made the most progress in clinical studies thus far. In phase II trials, Onconase exhibited promise in treating metastatic mesothelioma, but it underperformed in more sophisticated tests [21, 22] Despite this, it is still being studied in clinical trials as a potential antibiotic for the medication of various human papilloma-virus. The protein engineering has already been employed to develop variations with reduced energy of binding for cytosolic ribonuclease inhibitory protein (RI) to boost the therapeutic benefits of RNases in present and future clinical uses [23]. Ribonuclease inhibitory is a 50-kDa roughly circular enzyme that interacts to numerous members of RNase family and lessens cytotoxicity of RNase significantly [24]. The mutants of the living organism enzymes including RNase-1 with RNase-5 angiogenin, along with bovine pancreas RNase-A, have been engineered to retain catalytic properties while exhibiting lower binding to human RI [25] [26]. The analysis of crystalline structure of RNase-RI compounds using rational design methodologies enabled researchers to find critical positions at the nutrient interface. Amino acid changes at

these sites lower RI's binding affinity, which reduces RNase inhibitory activity. These modified human RNases have lately been studied as non-immunogenic, lethal missiles in association with cancer-targeting autoantibodies [27, 28].

Protein Engineered Proteases

Protease enzymes are the biochemical indicators for many existing targeted therapies, and they play an important role in trying to regulate essential biological mechanisms [29]. Protease enzymes are encoded in about 2% of the genetic code, and they are the cellular targets for several founded types of drugs. The wide range of medical disorders for which researchers are searching for modified proteases as medicinal enzymes is may be unsurprising. This would include leukaemia, vitreomacular adhesion as well as cardiovascular problems, numerous neurological diseases, and celiac disease [30, 31]. Chem and his co-worker were reported the configuration, kinetic characteristics, and configurations of arranged subtilizing proteases which cleave a variable regions in switch to destroy the active version of RAS. The RAS is a signalling protein which causes third part of all human malignancies when it is mutated. The active sites were engineered to exist reliant on a cofactor such as nitrite or imidazole and subtitles of protease were altered to create a connection among cofactor and substrate binding to provide high sensitivity and specificity for the RAS template strand. Specific proteolysis of activated RAS is the result of a two-step process in which sub-site associations boost productive cofactor attachment, allowing for cleavage. Furthermore, Proteases created in this manner cleave the RAS active sites in vivo, diminish RAS levels in a sensor system of bacteria, and decompose RAS in cultured cells of human. Even though these proteases are designed to host- active RAS, the basic structured principles are universal and can be used to a wide range of targets [32]. In accordance with Manasi's observations, the function of C1A cysteine proteases is suppressed by a newly developed way of re-engineered Mco-cysteine protease chimeric inhibitor that inhibits the function of the enzyme. An ultra-stable cyclic peptide scaffold, McoTI-II, was used to achieve this by grafting the cystatin beginning hairpin looping retained motif onto loop 1 of the scaffold. Because of the creation of multidisulphide linkages, the recombinantly produced Mco-CPI peptide was capable of transferring to papain with micro molar affinity and demonstrated outstanding thermo stability. Using an in silico technique based on homology modeling, protease docking, and the measurement of the free energy of contact, the mechanism of inhibition of Mco-CPI against typical C1A cysteine proteases was confirmed. Simulations of the Mco-CPI–papain combination using molecular dynamics software further demonstrated that the association is stable. The specificity of this McoTI-II counterpart was successfully oriented toward C1A cysteine proteases while maintaining a modest affinity [33]. The ability to use proteases in biotechnological and medicinal applications is improved by engineering precise high selectivity. Collagen disintegration, a therapeutic mechanism regulated by collagenases, is an important aspect of extracellular matrix remodelling and has been linked to a variety of clinical diseases when it is uncontrolled. Biswas et al. reported that cathepsin-K, a lysosomal enzyme, can break triple helix collagen fibers, while cathepsin-L cannot. By carefully altering proline-specificity and the glycosaminoglycans binding surface in cathepsin-L, researchers were able to give it collaenolytic properties. The proline-specific variant has significant prolylpeptidic substrates selectivity but is unable to cleave collagen. The proline-specific variant was able to breakdown type-I collagen in the context of chondroitin-4-sulfate after being given a GAG-binding surface (C4-S). The structural properties of proline-specific (1.4) and collagen-specific (1.8) variants can often be seen. Lastly, docking studies using a prolyl-peptidic substrates (Ala-Gly-Pro-Arg-Ala) at the protein surface and a C4-S compound at the GAG-binding site allow us to uncover critical structural characteristics that are crucial for cysteine cathepsins' collagenolytic activity [34]. Another study demonstrated that Metalloproteases and their isoforms have a wide range of biotechnological and medicinal uses. A new protease gene, nprB, was extracted and bioinformatics analysis was done on thermophilic bacteria, Streptomyces thermovulgaris. Sequence analysis and decoding of the nprB gene revealed a 178-aa open reading frame. The enzyme is classified as a thermal lysine like protease and a component of the M4 group of metalloproteases based on sequence homology, conserved features, and the PTF site. For functional and structural analyses, the ExPAsy server's ProtParam, SOPMA, and ProDom bioinformatics instruments have been used. A phylogram was also built to highlight the genetic links between nprB and its orthologs [35].

Protein Engineered urokinase

Urokinase has been tested as a synergic therapy for t-PA-induced fibrinolytic therapy [36, 37] Because of the consequences of urokinase in cancer, it is a focus for developing urokinase-specific inhibitors [38] or disrupting receptor connectivity [39, 40] Urokinase is primarily involved in Fibrinolytic stimulation on the cell surface, as well as neoangiogenesis [32] plasmin-mediated cytoskeletal breakdown [41, 42] and matrix metallic proteinase stimulation. These methods explain why urokinase is involved in cell migration, tumour metastasis, adhesion molecules, and cancer proliferation [43, 44]. Urokinase is a enzyme consists of 411 amino-acid with such a molar masses of 54 kDa that shares 40% of its amino acid sequence with t-PA [45] The EGF domains (1–49) that do not have a lysine transition state, a variable binder (132 to 158), a K domain (50 to 131), a proteinases region (159 to 411) that contains His204 Asp255, and Ser356428 enzymatic triad. Signalling through all of the urokinase receptors are aided by

phosphorylated on Ser138 and Ser303. On Thr18 urokinase is fucosylated, and on Asn304 it is N-glycosylated. The enzyme is synthesised as prourokinase, which is hydrolyzed into fibrin as well as other type of proteases around Lys158-Ile159, High - molecular - weight urokinase is a two-chain structure formed by the enzyme [46] [47] The EGF-K constituent of N-terminal and urokinase with a high molecular mass are both produced by further cleaving at Lys135-Lys136. The affinity of chimeric t-PA and urokinase regions for fibrin is low [48]. Because the P region of urokinase interacts with the both K domain of t-PA, Amediplase seems to have low fibrin selectivity. Because the P site of urokinase interacts with the both K sites of t-PA, amediplase has a low fibrin affinity. Across the other side, it penetrates the clot faster and also has a longer half-life. The single-chain variant of the Lys300His mutation has ten times lesser amidolytic efficacy than the two segments [49]. Dysregulation the plasminogen activator urokinase (uPA) and its coenzymes is linked to adverse outcomes in cancer patients. Authors had previously employed PET imaging with radioactively labelled specific antibodies against uPA (ATN-291) was developed to detect uPA signalling activity in a variety of cancers, but strong tumour comparison can only be obtained 24 hours after injection. The objective in this case is to take the original antibody and make an engineered antibody fraction (F(ab′)2) to reduce antibodies circulation time and linkages with the mononuclear phagocytes (MPS). F(ab′)2, ATN-291 was isolated and characterised using pepsin digestion and liquid chromatographic filtration. For PET imaging and fluorescent dyes cellular investigation, it was then coupled with Fluorescein isothiocyanate (FITC) or NOTA-Bn-NCS known as fluorescence microscopy or flow cytometry. F(ab′)2 ATN-291 demonstrated a higher targeting efficiency in MDA-MB about 231 uPA+ for uPA cells, as well as a faster plasma drainage speed than ATN-291, but its interaction with MPS was significantly reduced. In rodent tumour xenografts, radiolabeled F(ab′)2 ATN-291 absorption in MDA-MB 231 tumours was selective and persistent, with a tumor to blood ratio of 1.3: 0.8 (n = 4) at 2 hour post-injection using PET imaging. Throughout our work, radiolabelled F(ab′)2 ATN-291 was eliminated via both Hepatobiliary and renal and pathways. Radiolabelled F(ab′)2 ATN-291 was also used in test animals to detect uPA- fluctuation following tumour treatment [50]. The resent study revealed that although tissue plasminogen activator (tPA) has a high affinity selectivity Pro-tPA demonstrates catalytic activity for insoluble fibrin (IF) as urokinase (UK) against substrates apart from IF. UK has the benefit of being triggered only by IF; unfortunately, it binds IF only weakly. Researchers previously developed a monoclonal antibody (mAb) that detects a pit architecture that can only be found in IF. They created a recombinant protein of UK with humanistic 1101 Fab to deliver UK selectively to IF utilising a new anti-pit mAb, 1101, that does neither compromise thrombosis or fibrinolysis. Plasmin cleaves UK in two places. The locations in IF-containing nodules: Lys158/Ile159 and Lys135/Lys136. UK is activated by cleavage of the prior, but it is not released from the fusion since Between cleavages, activated UK is attached by S-S bonds [51].

Protein Engineered Staphylokinase

Staphylokinase is a tiny prokaryotic fibrinolytic accelerator produced by lysogenic *Staphylococcus aureus* that allows the bacteria to invade the cell of the human. The receptor of staphylokinase first was documented in 1948 [248] and generated recombinant in 1983. It was first described during most of the invention of streptokinase in 1933. Because it is immunogenic and also has a half-life of just 6 minutes, it is highly effective. Staphylokinase is a prospective thrombolytic for clinical use due to its large fibrinogen specificity, relatively cost effectiveness, and excellent clot permeability. Almost the entire pharmacologically significant natural thrombolytic, the enzyme has the shortest structure. It is made up of almost 136-amino acids in all, with 30% of them being charged. The protein is 15.5 kDa in size and is assembled into a single molecule. Staphylokinase is structurally and functionally similar with the region of streptokinase with the-grasp shape. Staphylokinase's immunogenicity precludes it from being re-administered, which is its main drawback. The primary epitopes identified by antibodies are 3 non-overlapping immunogenic areas on the surface of the protein. The immunogenicity of the preferred variant reduced to the less than 30% when synergistic mutagens-sis disrupted the recognized interactions protein fusion PEGylation, lipidification and glycosylation were used to create Staphylokinase’s variations with improved efficiency, shorter half-lives, decreased immunogenicity, as well as a reduced risk of reclusion [52].

Protein Engineered t-PA

In addition to fibrin-specific fibrinolytic therapy, t-PA plays a variety of roles in the brain. During therapeutic thrombolytic therapy, a high proportion of t-PA in the plasma might cause harmful side effects. t-PA enhances memory consolidation and recovery to brain injury by modulating cerebral flow of the blood barrier penetration in response to neurons activity [53] Blood vessel stiffness is affected by both the t-PA-PAI-1complex and t-PA. Dilatation is induced by the t-PA-PAI-1 complexes, whereas vasoconstriction is induced by t-PA alone. Dilation could have the benefit of allowing more blood to penetrate. Tenecteplase T-PA has the potential to cause blood to clot, which can be a disadvantage. T-PA is a protein complex of 527 amino acids that belongs to the class of trypsin-serine-proteases. It is available in two forms: type 1 t-PA and type 2 t-PA, both with a molecular mass of 63 and 66 kDa. Type 1 t-PA is the more common of the two. One of the objectives of t-PA enzyme modification was to increase its half-life [54].

Just the K2 and P sites of a deletion mutant reteplase are preserved and the tetra-alanine mutation is present. The reduction of MR binding and lowered binding to LRP1, that are accountable Improves half-life for the short half-life of a few 4.5 minutes. Following LRP1, MR, galectin, and maybe the asialoglycoprotein receptor, LRP1 plays a crucial role in evacuation. Gp is very similar to LRP1 and can be involved in t-PA elimination in the kidneys. On LRP1, complement repetitions of group include binding affinity both raw t-PA and t-PA-PAI-1 complexes were tested. The antagonists protein can reduce adhesion (RAP), that works simultaneously allosterically and by interfering for a component of the binding domain [55] Aspartates surrounding Calcium 2^+ ions link accessible lysines in LRP1's ligand-binding pathway. The LRP1 active sites of t-PA and desmoteplase can be located in the E, F, and K1 subdomains.The Tyr67Gln mutation inhibits binding by introducing an additional oligosaccharide, which prevents LRP1 engagement. The impacts of LRP1 association govern t-pleiotropic PA's actions in neurobiology, but they also contribute significantly to t-neurological PA's side effects. employed therapeutically. Whenever t-PA is utilised to treat hypoxic - ischemic strokes, all of these impacts on the neurological system are reinforced. The large oligosaccharide linked to Asn117 has been shown to join the MR. NMDAR-mediated neurotoxic effects is reduced when K2 is deleted, as were the mutations in the positive charge loop. Proteolytic activity, on either side, is not required for NMDAR stimulation, hence the precise relationship remains unknown. Desmoteplase is not neurotoxic because it lacks a lysine binding site [17]. NMDAR is activated solely by the single feature of t-PA. t-PA interacts with Annexin A2-S100A10 via C-terminal lysines, indicating that the lysine active site on the K2 domain is the most likely binding site for the protein. A2-S100A10, also known as Annexin A2, connects plasminogen to cell membranes and serves as a source of fibrinolytic activity [56].

Protein Engineered streptokinase

Streptokinase is a prokaryotic exocellular plasminogen stimulator that was initially discovered in 1933 [56]. It is basically formed by -hemolytic streptococci strains, that use it to overcome the patient's defensive fibrin membrane and promote bacterial colonisation and metastases. There are various streptokinases of various streptococci that change in structure, however the only streptokinase variation now used as a thrombolytic agent is from group C streptococci and is insensitive to fibrinogen. Considering its modest in vivo half-life about 10 minutes and much cheaper cost, the primary drawbacks are the lack of fibrin selectivity and immunology owing to the microbial origin of the material [57]. Streptokinase has a molecular mass of 47 kDa and is a monomeric kinase. Streptokinase is composed of amino acids, and the physiological unit forms an isomeric protein with a molecular size of 47 kDa. The pH maximum is somewhere between 7.3 and 7.6, and the isoelectric is 4.7. Streptokinase includes three -grasp domains and as revealed by magnetic resonance and spherical dichroism investigations, as well as a crystallographic examination of the micro plasmin-streptokinase complexes. Streptokinase's fibrinogen specificity was improved using a variety of methods. The Del domain prohibited streptokinase from interacting with plasminogen in its contracted configuration. However, it produced the streptokinase-plasmin compound entirely and could only stimulate plasminogen attached to the fibrin surface in the extended position. Streptokinase was transformed into an activator that looked like Staphylokinase [58, 59]. Employing Streptokinases specialised for fibrin from a variety of Streptococcus strains or altering promoter domains to merge their functions was an additional option. The fibrin selectivity of these novel compounds was comparable to or even greater than that of t-PA, while retaining the original activity. Streptokinase's half-life has been increased through various engineering efforts. The strategies were based on the realization that Streptokinase is catalysed non-physiologically by plasmin into three peptide chains (residues 1–59, 387–414, and 60–386), resulting in a decrease in activity. Using mutagenesis to inhibit hydrolysis at the indicated locations, the half-life was enhanced by 21-fold without compromising activity. Streptokinase variants with better thrombolytic performance, lower immunogenicity, and longer half-lives were created using alternative techniques such as combinatorial mutagenesis, glycosylation, lipidification and PEGylation [59, 60]

Streptokinase's acylated compound form, known as anistreplase, is a particularly successful version. Anistreplase is a streptokinase-plasminogen enzyme that has had the active site of plasminogen inhibited by anisoylation. P-anisic acid is hydrolyzed and eliminated in vivo, leaving an enzyme-active plasminogen promoter. The half-life of the variation was extended roughly 10-fold while maintaining the original function and antigenicity of streptokinase [61].

Table 1: Comparison of thrombolytics in terms of structural properties

Fibrinolytics	Domains	Action method	Affinity for Fibrin	Selectivity for Fibrin	Inhibition Issue
Streptokinase enzyme	alpha, beta, gamma	In-direct Method	No	No	Decreased
Urokinase	K, EGF, K,	Direct	No	Decreased	Increased

Enzyme		method			
Tissue- plasminogen-activator	EGF, P, F, K1,K2	Direct Method	Intermediate	Intermediate	Increased
Desmetoplase enzyme	EGF, P, F,	Direct Method	Increased	Increased	Decreased
Staphylokinase enzyme	alpha	Indirect Method	No	Increased	Decreased

Protein Engineered Collagenase

Collagenase is a one-kind of protease susceptible of hydrolyzing native collagen in a specific manner. It features a saddle-shaped three - dimensional structure, and the active site contains a Zinc moiety. The chains of two histidine residues, one glutamate, and a water molecule tetrahedral coordinated the Zinc component, with the molecule of water hydrogen-bonded to some other glutamate protein. Microbial collagenases are metalloproteases that may breakdown extracellular matrix, making them essential virulence agents. *Clostridium histolyticum*-produced collagenase is being used to disintegrate burn scars instead of harsh surgical treatment [62]. Debridement is the process of removing diseased or damaged tissue in order to provide enhanced treatments and active restoration. Medical and physical debridement procedures are less precise and painful. This enzyme destroys necrotic decaying tissues in a painless and selective manner. It promotes wound healing by releasing collagen-derived proteins, which boost macrophage chemotaxis and cytokine release, promoting wound healing. A widely established enzymatic debridement regimen is Collagenase treatment, which contains Clostridial collagenase as well as some few other general proteases. It's administered directly to scars or skin lesions to hasten healing and eliminate undesirable tissues [63]. Clostridial collagenase ointment (CCO) seems to be the only FDA-approved therapy for catalytic postoperative wound in severe burns in the United States, and it is successful in reducing wound recovery time and discomfort while lowering infection risks [64]. Dupuytren's-disease is a fibro-proliferative disease characterised by the formation and gradual rigidity of isolated parts of the palmar aponeurosis, limiting hand usage in later stages. The formation of collagen-rich cords, as well as gradual finger flexion, takes place. It's a life-threatening disease with a wide spectrum of symptoms and clinical presentations. Injections of Clostridium histolyticum collagenase have been suggested as a possible first-line treatment for DD [65]. Adult men with Peyronie's disease (PD) are given intralesional injections of a mixture of class-I and class-II collagenases of C. Histolyticum [66].

Protein Engineered Dismutase

Superoxide anion is produced by all aerobic organisms as by-products of regular aerobic metabolism and as extrinsic stimulus reactions. Superoxide anions, peroxides, and hydroxide ions are examples of these. These operate as oxidative stress mediators. Inflammation, carcinogenesis, neurological diseases, and ageing can all be caused by reactive oxygen species. To combat the effects of ROS, cells have the ability a complex set of antioxidant defence system. Among the most important defence systems is Superoxide Dismutase (SOD), which is found in practically all cells. SOD is a metalloenzyme that is found all over the body. The active site is securely held in place by an 8-stranded beta-barrel, which itself is held in place by the two surface loops. In a back-to-back arrangement, two subunits are securely linked via mostly hydrophobic and very few electrostatic contacts. Lawakwa's team were reported that crucial factor in the start of amyotrophic lateral sclerosis is the creation of aggregates of superoxide dismutase 1 (SOD1) inside motor neurons. In crowded situations, such as inside a cell, the thermal stability of the SOD1-barrel has been demonstrated to deteriorate, however it is unknown how the thermodynamics of crowding-induced protein instability relate to SOD1 aggregation. Authors investigated the impact of a proteins crowder, lysozyme, on SOD1-barrel fibril aggregation formation. Even in moderately crowded conditions, the production of SOD1 aggregates is slowed. Temporary diffusional contacts with lysozyme, it turns out, have no effect on the framed structure of the SOD1-barrel, but they do stabilise an alternate excited "transparent" state. Moreover, crowding has the net impact of favouring species that are not on the aggregation pathway, which explains why aggregation is slowed in a crowded environment [67]. Another study of Boracco described that CYP154C5 is a P450 monooxygenase from Nocardia farcinica that can hydroxylate a wide spectrum of steroids with strong regio- and stereoselectivity at the sixteen locations. Employing protein engineering and substrates modifications based on the crystalline structure of CYP154C5, the enzyme's steroid hydroxylation regioselectivity was adjusted. In addition to 16hydroxylation, progesterone transformation by mutant CYP154C5 F92A resulted in the creation of the equivalent hydroxylated product 11-alpha deoxycorticosterone. This modified regioselectivity appears to be the result of a different steroid binding mode in mutant F92A's active site, according to MD modelling. The introduction of water to the protein surface generated increased uncoupling in this mutant, according to the MD simulation. Furthermore, in the transformation of 5androstan3one, which lacks an oxyfunctional group at C17, wild - type strain CYP154C5 showed exclusive 15hydroxylation.

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Collectively, these findings provide important information on the structure link of this cytochrome P450 monooxygenase involved in steroid hydroxylation [68].

Table 2: Implementation of engineered therapeutics in summary

Therapeutic Enzymes	Modification	Applications
E-Hybrid and BoNT/A Enzyme	BoNT/E protease domain-fusion with BoNT/A translocation and binding-domain	Higher pain- attenuation
GRRH-hybrid Fusion of BoNT-D-protease Enzyme	GRRH-binding-peptide domain	Inhibit the growth of pituitary hormone hyper secretion in the animal prototype of acromegaly
t-PA Tenecteplase-TNKase Enzyme	R298A/T103N /K296A/ R299A H297A/ N117Q /	In vitro reduction inhibition as well as improved half life
BoNTs	Used as engineering of host specificity	Removed Chronic problem
Urokinase Enzyme	No mutation	Used as Fibrinolysis for medication of pulmonary diseases like embolism
BoNT-BMY Enzyme	Mutations improving connection to host human synaptotagmin-II, modifications of the lipid-binding coil	Improved effeciency
BoNT-LC Enzyme	Vector asserted transgenic BoNT-LC complex	Permanent, demanding, and have ability to control, BoNT-LC manifestation in various Neurons category
BoNTs Enzyme	Ligation to tools in delivery targeting BoNT into special types of cells	Give relief in pain including neuropathic pain and remove inflammation
BoTIMs Enzyme	Longer BoNTs adjustments inactive LC-A and LC-E	Prolonged effect on a variety of pain disorders, including chronic pain
BoNT-A Enzyme	Stapling of Protein permitting in vivo reassembly of BoNT-A	Improvement of neural modulating Component
BoNT-LC Enzyme	Mutation of LC	Used to cleave syntaxin
t-PA Enzyme	N117	Removal of fibronectin along with epidermal development component domain Boosted half-life
BoNT-A Enzyme	V258P, L256E and A308L and V258P mutation	particularity, rather cleaving SNAP-23 network to remarkably restrict Pathogens secretion

Protein Engineered BONTs

BoNTs are a type of neurotoxin generated by *Clostridium botulinum* and other Clostridium spp. strains, including such *Clostridium baratii* and *Clostridium butyricum*. The BoNTs are recognised as one of the "dirty dozen" chemicals that could be used as biological weapons [69] Botulism is a disease caused by BoNT intoxication result of the production of formulated food tainted with botulinum toxin or infection with *C. botulinum* choices rather than solely Infant caused by the

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bacterium and intestinal toxaemia are caused by the spread of *C. botulinum* spores and toxigenic in the gastrointestinal system. Botulism is more commonly caused by wound exposure. Botulism is characterised by a flaccid periphery paralyze caused by neurotransmitters inhibiting discharge at nerve terminals, which can be deadly if not treated in a care unit. Botulism therapy is primarily symptomatic, with severe cases requiring intensive care and mechanical ventilation [70].

The translocation site on HCN facilitates LC delivery from across vesicular membrane in allowed to penetrate the neuronal cytosol on further acidification, while the HC 1/2 domain, which consists of two sub-domains named as HCCn domain and HCCc domain, is appropriate for different adhesion prior to endocytosis, Preceding endocytosis, the toxin binds to the presynaptic membrane of neuronal synapses, causing the poison to be released from the presynaptic membrane of neural connections [71, 72] Using BONTs to treat hyperactive muscles, Dr. Allen Scott, an ophthalmologist on the lookout for a new way to treat his patients, discovered a new approach to treat his patients. Dr. Allen Scott and a microbiologist collaborated to treat hyperactive muscles. BoNTs have been licenced by the FDA for a variety of illnesses involving excessive muscular contractility. BoNTs are traditionally used to treat motor symptoms like dystonia and spasticity [73], but they're also utilised to treat glandular hyper secretion like hyperhidrosis and sialorrhea [23]. It has also been shown that BoNT modulates the sensory feedback loop to the central nervous system (CNS), resulting in analgesic effects of the drug. The toxin exhibits diverse effects in inhibitory and excitatory neurons, making it a one-of-a-kind neural modulation therapy [74].In persistent migraines, BoNT has been shown to have analgesic effects [75]. BoNTs are increasingly being employed in a growing variety of medical disorders involving muscle spasms. The therapeutic usage BoNTs in pain reduction has attracted much interest, resulting in several of the novel applications in pain-related clinical disorders. By blocking synaptic vesicle fusion and changing the transient receptor potential (TRP) of muscle spasm trans membrane receptors at the neuronal cellular membranes, it is possible to treat muscle spasms and other neurological disorders, BoNTs actually hinder the release of migraine neurotransmitters including such glutamate, calcitonin gene-related peptide and substance (SP) [76]. BoNT/A inhibits nociception by blocking signal transduction pathways fusion of ion channels inside the nerve terminating membrane of peripheral trigemino-vascular neurons, according to Aoki et al. The impacts of BoNT/A on migraine headaches are driven by changes in vesicles transport, neurotransmitter release, and proinflammatory polypeptide release, along with changes in the expression of key ion channels and receptors in nerve terminals [77].

Protein engineered BoNTs for Improved Activity in Humans

Mutations at critical regions of the HC, HCN, and LC can largely be used to modulate BoNT activity [77].To increase the toxin's efficacy, mutagenesis experiments have traditionally been designed to make HC-A and HC-B have a better time attaching to gangliosides as well as polypeptide receptors. Tao et al. found polymorphisms in BoNT/B that improve the interaction to humans synaptotagmin-II in a selective manner (Syt-II). Modified BoNT/B inhibited neurotransmission 11 times far beyond wild type BoNT/B in cultured neurons [78] In vitro studies have also been carried out in order to improve the activity of BoNT/LC B on VAMP. However, comprehensive BoNT/B bearing the similar mutation did not demonstrate increased efficacy in either fibroblast tests or in vivo models [79]. BoNT's LC has already been adjusted to keep syntaxin 1 cleave. The therapeutic importance of the BoNT-C and the function of syntaxin 1 in synaptogenesis have both been established [80]. In human or recombinant mice, modified rBoNT/B1 toxins demonstrated increased effectiveness and enhanced affinity for hSyt2 and hSyt1. The higher affinity for human receptors resulted in increased activity in all animals that generated human Syt isoforms. As a result, tailored rBoNT-B1 toxins with higher affinity for hSyt2 and hSyt1are a suitable option to BoNT-A1 therapy, especially for patients with anti-BoNT-A1 antibodies [81]. Core-therapeutics were developed by Tang and his colleagues using a sortase-based technique for binding cloned BoNT enzymes and a translocation component to receptors that govern the distribution of SNARE-cleaving proteases into specific cell types. A game-changer in specific BoNT activities such as pain management is the delivery of a SNARE protease to specific neural subsets or non-neuronal tissues using this method of delivery. Because they are linked to specific cell subtype ligands that block the function of pro-inflammatory cytokines or key pain neuropeptides, these novel transgenic BoNTs hold great potential for in vivo studies of diabetic neuropathic pain in animal models of the disease [82]. BoNTs have been used to develop a novel protein family (target secretion inhibitors (TSI), also known as directed vesicles exocytosis-modulating protein (TVEMP)), which consists of three fundamental domains, with each contributing to the overall activity of the molecule. Target secretion inhibitors (TSIs) are a type of protein that inhibits the secretion of target secretion inhibitors (TSI). The LC domain of one particular BoNT toxin type confers SNARE cleavage capabilities depending on the BoNT toxino-type. The LC's intracellular translocation capacity is provided by the HN domain, and the binding domain is typically a peptides or proteins that connect with receptors of choice on the target tissue. The endopeptidase subdomain is used in this novel strategy to modulate the intracellular activities of target cells while also suppressing their

secretion mechanisms [83]. Because of the toxin's modular nature, there have been numerous chances to transform BoNTs into non-neuronal therapies that are more efficacious, less toxic, or transcoded Nonetheless, learning more about BoNT engineering and how to use it to expand processes and capabilities therapeutic interventions will contribute in the development of innovative approaches to treating acute and chronic human pain. Engineered BoNTs are also expected to make significant advancements in neuro-protective methods, neuronal proliferation, and the therapy of hyper secretory and hormonal diseases [84].

II. CONCLUSION

The enzyme based therapies particularly advancement in designs, throws up endless possibilities for developing extremely effective and specialized treatments. Whether the enzyme is the treatment itself, is still important in the formation of active drug, or works as a detection device for clinical biomarkers, these biomolecules are a powerful and almost endless resource for therapeutic applications in protein engineering as well as genetic variability. Over the last two decades, therapeutic enzyme engineering approaches have usually supported advancements in protein engineering. our review summarizes the current studies showing the advancements and limitations in using these methods to therapeutic enzyme engineering.

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