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Research Article

IMMOBILIZATION OF RECOMBINANT TRYPSIN ON MAGNETIC PARTICLES FOR CONTROLLED PROTEOLYSIS AND CONFIRMATION OF ITS ACTIVITY

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ABSTRACT

Controlling the enzymatic digestion during the production of biologics is one of the key issues associated with proteolytic enzymes such as trypsin. Uncontrolled enzymatic activity results in generation of impurities which puts tremendous pressure on the purification tools to obtain therapeutic level of purity. Even though the inhibitors such as phenylmethyl sulfonyl fluoride, Soyabean trypsin inhibitor, etc. are commonly used in production strategies, complete inhibition is not achieved, thus laying a platform for the use of immobilized trypsin. The use of immobilized enzyme provides better control on enzymatic reactions and also on the traces of residual trypsin in the finished product. In the current work, recTryp has been immobilized using N-Hydroxysuccinimide (NHS) activated Biotin and Streptavidin coated magnetic (SCM beads). The coupling between recTryp and NHS activated biotin was performed at a ratio of 1:15 (w/w) in the presence of sodium carbonate buffer, pH 9.5. The excess biotin was removed by dialysis against PBS, followed by coupling between SCM beads and Biotinylated recTryp. The non-specifically adsorbed enzyme on the beads was removed by extensive washing against PBS. The activity of the same was identified by performing BSA digestion assay. The immobilized system can also be used in the insulin production.

INTRODUCTION

The process of recombinant DNA technology has opened up varied possibilities of generating biologics. The recombinant DNA technology has been used extensively in manufacturing of therapeutics as well as commercially important enzymes. The enzymes such as Trypsin, Carboxypeptidase B, Enterokinase, etc are considered as critical enzymes as they are used routinely in the manufacturing of biologics such as human insulin and analogs and other proteins of therapeutic importance cloned as fusion protein (Wolfgang Kemmler 1971). The enzyme Trypsin is a serine protease and is commonly used in proteomics and it cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. Trypsin is typically isolated from porcine or bovine pancreas is used extensively in the manufacturing of insulin and enzymes such as Carboxypeptidase B. With the advent of rDNA technology, recombinant forms of enzymes are available and hence recombinant trypsin (recTryp) can be used successfully in the manufacturing of Trypsin, Carboxypeptidase B, Enterokinase, etc.Immobilized enzymes have been widely used in the processing of variety of products. New strategies are continuously emerging for the formation of diverse immobilized enzymes having superior efficiency and usage (Taylor *et al*. 2015). Immobilization often stabilizes structure of the enzymes, thereby allowing their applications even under harsh environmental conditions of pH, temperature and organic solvents. Techniques for immobilization have been broadly classified into four categories, namely entrapment, covalent binding, cross-linking and adsorption. A combination of one or more of these techniques has also been performed. Covalent binding is an extensively used technique for the immobilization of enzymes. The functional groups extensively investigated are the amino, carboxyl, and the phenolic group of tyrosine. Enzymes are covalently linked to the support through the functional groups in the enzymes, which are not essential for the catalytic activity. Efforts have been made in the direction to magnetize the biocatalyst either by directly binding the enzyme on magnetic materials like magnetite, magnetic micro particles or magnetic beads. Also, it can be achieved by co-entrapping magnetic material which can be easily be recovered using an

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external magnetic field as these matrix provides the rapid and ease of separation of enzyme once the external magnetic field is applied (Iype *et al*. 2016)· (Mohamed *et al*. 2017).

Immobilization of various ligands with the aid of bio-specificity is widely used technique for immobilization. These include examples like Immobilized affinity tags such as GST, FLAG, maltose, lectins, heparin, chitin and streptavidin for purification of proteins. One such bio-specific immobilization technique was considered in the study of streptavidin based immobilization. Streptavidin,~ 60kDa protein purified from Streptomyces avidinii, has an extraordinarily high affinity for biotin (Figure 1)(Suter *et al*. 1988) and is used extensively in molecular biology and bio-nanotechnology as a high-affinity biotin-binding agent. The bond formation between Biotin and Avidin is very rapid, and once formed, it is unaffected by extremes in pH, temperature, organic solvents and other denaturing agents. Exploitation of the streptavidin–biotin interaction is extremely valuable in a variety of biotechnological applications (Sorenson, Askin, and Schae 2015).

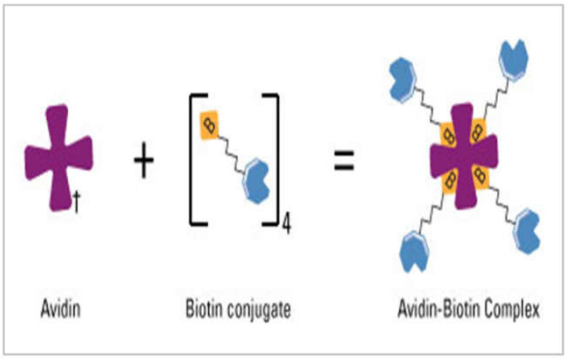

Fig 1 Avidin Biotin interaction

Streptavidin beads have been used to display immobilized biotinylated antibodies to detect the existence of certain antigen(Lu *et al*. 2011). Many of the kits related to molecular biology, proteomics, cytological studies, immunological assays are based on this type of chemistry where the interaction between two are used as a tool in non-radioactive methods of purification, detection, immobilization and labeling.

Immobilization of trypsin on biotinylated cellulose beads with sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) streptavidin and biotinylated trypsin were sequentially adsorbed that resulted in 32 U/mL of immobilized trypsin. The outcome of this study illustrates that cellulose beads can be biotinylated for use as bio-selective support(JANOLINO and SWAISGOOD 2010).

For easy and efficient binding of biotin and enzyme which is to be immobilized, presence of reactive groups plays significant role. Amines are the most commonly targeted functional groups for biotinylation because of the abundance of lysine side chain ε-amines and N-terminal α-amines. In the current study, N-Hydroxysuccinimide (NHS) activated biotin was used for biotinylation of recTryp as NHS esters readily form stable bonds with primary amines, and the reactive group is easily incorporated and stabilized. The mechanism of EDC-based crosslinking of antibodies was proposed by Nakajima and Ikada. The NHS esters produced by EDC/NHS and EDC/sulfo-NHS were proposed to be more stable than the O-Acylisourea ester produced by EDC(Vashist 2012), (Scientific and Handbook 2012). The current work describes about immobilization of trypsin on SCM beads via biotinylation of the enzyme. Post immobilization the retained esterolytic activity was confirmed using BSA digestion assay. The immobilized system can also be used in the insulin production.

MATERIALS AND METHODS

Materials

Reagents/Chemicals

Standard streptavidin, Standard BSA, N-α -Benzoyl-l-arginine ethyl ester (BAEE) (Sigma Ald.), NHS activated biotin (Sigma Fluka), recTryp (3.4.21.4), Human Proinsulin (HPI), Human Insulin (HI), Carboxypeptidase B (CPB) (Biogenomics Pvt. Ltd (Thane, India)). Streptavidin coated magnetic particles (Thermo Scientific), Dimethylformamide (DMF), Sodium carbonate, Sodium bicarbonate, Monosodium phosphate, Disodium phosphate, Sodium chloride, Tris-HCl, Glycine, Acetone (Merck), β-Mercaptoethanol, SDS, Acryalamide, Bisacryalamide, APS (Ameresco).

recTryp stock 10 mg/mL, BSA stock 1mg/mL, NHS-Biotin stock 0.01 mg/mL, Streptavidin stock 0.86 mg/mL, Coupling buffer (100 mM Carbonate, pH-9.5), PBS (50 mM Phosphate, 150 mM NaCl, pH 7.4).

Instruments and Equipments

UV-Visible spectrophotometer (Shimadzu 1800, LabIndia 3000^{+}), Biorad SDS-PAGE apparatus, pH meter (EuTech), Rocker, Stirrer (Acensions Innovation), and Vortex (Remi).

Miscellaneous

Dialysis tubing-10 kDa MWCO (Thermo scientific)

METHODOLOGY

Complex Formation of Biotinylated recTryp and Free Streptavidin

Biotinylation of recTryp

Spectrophotometrically recTryp concentration was adjusted to 1 mg/mL; NHS-Biotin was dissolved in DMF at a concentration of 0.01 mg/mL. A solution of 1 mg/mL recTryp was aliquoted for biotinylation of the protein and mixed in a molar ratio of 1:40 of recTryp to Biotin. recTryp was incubated overnight at 4^{o}C with NHS-Biotin for biotinylation in presence of coupling buffer. After the incubation, the biotinylated molecules were dialyzed against PBS to get rid of unbound/free/excess biotin in the reaction. The analysis of biotinylated molecules was carried out on SDS-PAGE with appropriate controls and protein marker. Also, the recTryp activity assay was carried out before and after biotinylation.

Binding between biotinylated recTryp and free streptavidin

Biotinylated recTryp was then allowed to form complex with 0.1 mg/mL of free streptavidin in presence of phosphate buffer pH 7.4. The biotinylated protein complexed with streptavidin was analyzed using SDS-PAGE.

Immobilization of biotinylated recTryp using Streptavidin coated magnetic beads

Binding between biotinylated recTryp and SCM beads SCM bead slurry of about 0.1 mL was taken to obtain 0.05 mL of beads for conjugation. These beads were then washed 10 times with PBS by vortexing for 30 seconds in order to equilibrate the beads. One mL of Biotinylated recTryp was added to the washed SCM beads. The reaction mixture was then incubated for 4 hours at room temperature under stirring condition. Post conjugation extensive washes were carried out with PBS for complete removal of nonspecifically bound enzyme. These washes were then analyzed and assayed for residual recTryp using SDS-PAGE and BAEE activity assay.

BAEE substrate Assay of recTryp activity

The Activity of recTryp was calculated using esterolytic activity assay (United States Pharmacopoeia). BAEE stock of 8 mg/mL was prepared in 0.067 M phosphate buffer pH 7.6, and using BAEE stock optical density of BAEE substrate buffer was adjusted between 0.575-0.585 at 253 nm using UV-Visible Spectrophotometer. Enzyme concentration was adjusted to 1 mg/mL spectrophotometrically and 0.2 mL was added in 3 mL of BAEE substrate buffer and rate of change in absorbance per minute was noted. The activity was calculated using following formula,

USP units / mL= $\Delta A*df/ (0.003*1*0.2)$
USP units/ mg = $\Delta A/ (0.003*1*mg)$

Where,

ΔA is rate of change in absorbance,
df is Dilution factor,

0.003 is standard average rate of change in absorbance per min for BAEE.

The recTryp, biotinylated recTryp and washes were subjected to BAEE assay as per above mentioned procedure and Enzymatic digestion reaction of BSA using decreasing concentration of free recTryp are tabulated in Table 1.

Table 1 Enzymatic digestion reaction of BSA using decreasing concentration of free recTryp and immobilized recTryp

Sr. No.	BSA mg	recTryp in µg	Units	Vol. of recTryp µL
1	0.2	100.00	360.00	20.00
2	0.2	50.00	180.00	20.00
3	0.2	25.00	90.00	20.00
4	0.2	12.50	45.00	20.00
5	0.2	6.25	22.50	20.00
6	0.2	3.13	11.25	20.00
7	0.2	1.56	5.63	20.00
8	0.2	0.78	2.81	20.00
9	0.2	0.39	1.41	20.00
10	0.2	0.20	0.70	20.00
11	0.2	0.10	0.35	20.00
12	0.2	0.05	0.18	20.00
13	0.2	0.02	0.09	20.00
14	0.2	0.01	0.04	20.00
15	0.2	0.01	0.02	20.00
16	0.2	0.00	0.00	0.00
17	0.2	-	-	10µL beads

Enzymatic Digestion (ED) Reaction of HPI in presence of CPB

ED reaction of HPI can be used as an assay for immobilized recTryp as well as a sensitive assay to monitor the generation of HI from HPI using recTryp present in the trace amount. As the BAEE assay was not much sensitive to detect residual recTryp in the washes, enzymatic digestion of HPI was carried out. 0.01 mL of Immobilized recTryp was incubated with HPI in the Presence of CPB at pH 7.4, at room temperature.
Various sets of enzymatic digestion reactions are represented in Table 2.

Table 2 ED reaction of HPI to HI

Sr.No	Sample Name	HPI	HPI+CPB	Std. recTryp	Biotinylated recTryp	Immobilized recTryp (Beads)	Washes
1	Substrate control	100 µL	--	--	--	--	--
2	-ve control	--	100 µL	--	--	--	-
3	+ve control	--	10 µL	8 µL	--	--	--
4	Biotinylated	--	100 µL	--	0.2 µL	--	--
5	Washes	--	100 µL	--	--	--	10 µL
6	Test Sample	--	500 µL	--	--	20 µL	--

SDS-PAGE analysis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) analysis of post ED samples

All the samples were processed under non-reducing conditions and were loaded on 15% Polyacrylamide gel for streptavidin complex formation and sample of ED reactions were loaded on 18% Polyacrylamide gel. The detection was carried out using silver staining.

RESULTS

SDS-PAGE Analysis of Biotinylated Proteins by Electrophoretic Mobility shift assay

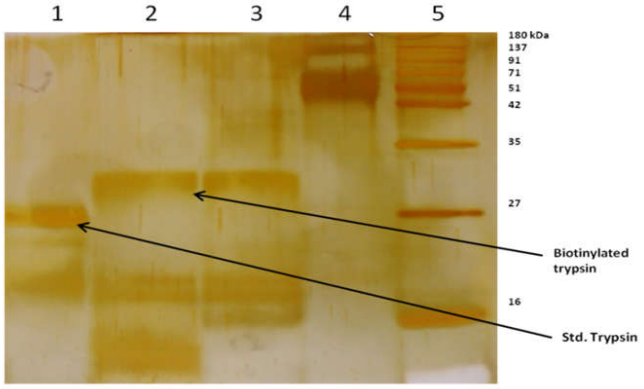

Fig 2 15 % PAGE, stained with silver stain, under Non-reducing condition showing samples of pre and post biotinylation of recTryp

Lane no.	Sample Name
Lane 1	Std RecTryp
Lane 2	Biotinylated recTryp
Lane 3	Bt-RecTryp-Streptavidin complex
Lane 4	Streptavidin standard
Lane 5	Protein marker

In figure 2, it is observed that the band present in the 2nd lane is slightly upward than the band observed in lane 1. The slight shift in electrophoretic mobility is due to the increase in molecular weight of recTryp after biotinylation due to binding of biotin to the recTryp. The method relies on complex

formation between a biotinylated macromolecule and a streptavidin probe resulting in an electrophoretic mobility shift of the complex detectable by SDS-PAGE. (Sorenson, Askin, and Schae 2015)

BAEE Based Activity assay of Rectryp before and after Biotinylation

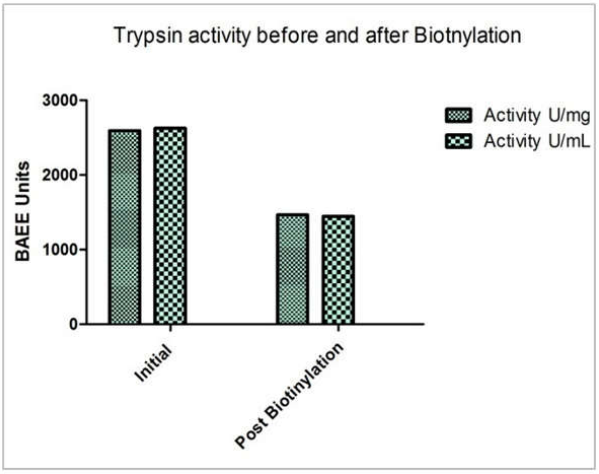

Fig 3 Graphical representation of decrease in the activity of recTryp before and after biotinylation

Table 3 Activity of recTryp before and after biotinylation

	Activity Units/mg	Activity Units/mL
Initial Units	2594.68	2626.67
Units after Biotinylation	1466.76	1446.67

Activity assay was carried out to check the activity of the biotinylated recTryp (Table 3). As described in figure 3, it is observed that post biotinylation approximately 56% esterolytic activity of recTryp is retained. The resultant reduced activity could be due to the NHS activated biotin moiety which targets 1^{o} -NH_2 groups in the proteins. The 1^{o} -NH_2 groups in the catalytic triad also involved in the covalent bond formation with NHS activated biotin which causes reduction in the activity post biotinylation.

SDS PAGE Analysis for Complex Formation between Biotinylated Rectryp and Streptavidin

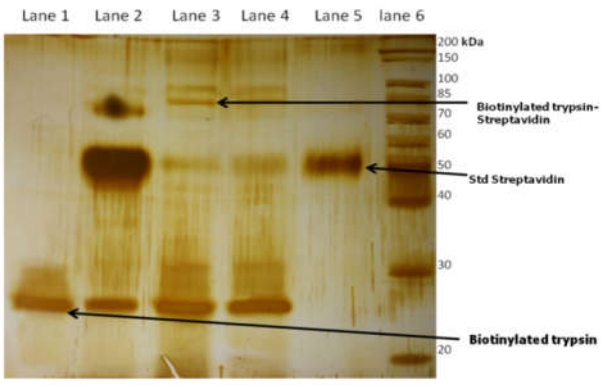

Fig 4 15 % PAGE, stained with silver stain, under Non-reducing condition showing biotinylated recTryp-streptavidin complex

Lane no.	Sample Name
Lane 1	Biotinylated recTryp
Lane 2	1:1 of Biotinylated recTryp and Streptavidin
Lane 3	1:10 of Biotinylated recTryp and Streptavidin
Lane 4	1:100 of Biotinylated recTryp and Streptavidin
Lane 5	Std. Streptavidin
Lane 6	Protein marker

As streptavidin is tetravalent and four biotin moieties can bind to each streptavidin, hence four biotinylated molecules should bind to one streptavidin molecule. There may be possibility that either one or two biotinylated molecules binds to streptavidin and forms the complex which is represented in the figure 4.

Band near~ 85 kDa indicated the complex formed between single biotinylated recTryp and free streptavidin. There might be formation of multi-biotinylated molecules complexed with streptavidin, molecular weight of which could be more than 100 kDa, which are not clearly visible due to the lower abundance of the event. At lower ratio of biotinylated recTryp and streptavidin the complexation obtained was very less as a result unbound streptavidin yielded a thick band is observed. At 1:1 ratio, streptavidin is remaining unutilized due to lesser amount of binding molecules, hence showing thick fragment at the position coinciding with the Std. streptavidin.

The above results confirm the formation of biotinylated protein-streptavidin complex; hence immobilization of recTryp on streptavidin coated magnetic particles was done successfully.

The activity of biotinylated recTryp–streptavidin complex was checked using enzyme digestion reaction.

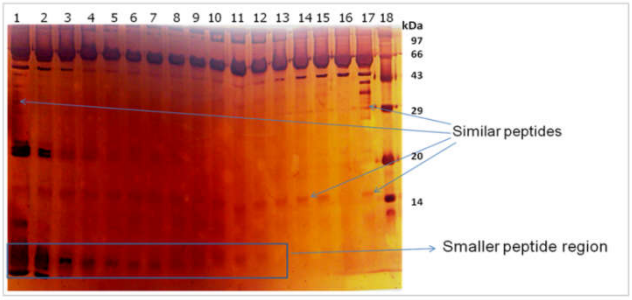

Fig 5 18% SDS PAGE, Silver stained, showing BSA digested with decreasing concentration of recTryp.

Lane no.	Sample Name
Lane 1 to 15	BSA digested with decreasing concentration of recTryp
Lane 16	Std BSA
Lane 17	BSA digested with Immobilized recTryp
Lane 18	Low range molecular weight marker

Protease activity of immobilized recTryp was investigated using a BSA as a model protein substrate. In figure 5, lane 17 shows bands near the intact BSA band indicating the slow digestion of BSA with immobilized recTryp beads. Lane 1 to 15 shows digestion of BSA with decreasing concentration of std. recTryp and digesting BSA that forms smaller sized peptides at the bottom. The amount of peptide generated was found to be decreased as the recTryp concentration decreases. If we compare the activity of std. recTryp of lowest

concentration and immobilized recTryp, the later one showed very less activity even though the digestion period was kept constant.

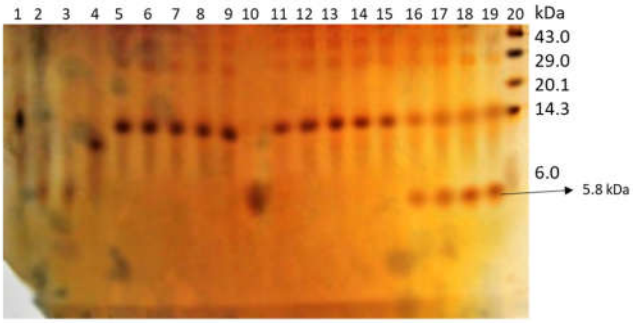

Fig 6 SDS PAGE analysis of samples described below in tabulated form, loaded on 18 % polyacrylamide gel, and stained using silver staining procedure.

Lane No.	Sample ID	Lane No.	Sample ID
Lane 1	HPI + CPB w/o recTryp (-ve ctrl)	Lane 11	Std. HPI
Lane 2	HPI + CPB + recTryp (+ve ctrl)	Lane 12	Bead ED 0.5 hr + TFA
Lane 3	Biotinylated recTryp	Lane 13	Bead ED 0.5 hr w/o TFA
Lane 4	Wash 1	Lane 14	Bead ED 1 hr + TFA
Lane 5	Wash 2	Lane 15	Bead ED 1 hr w/o TFA
Lane 6	Wash3	Lane 16	Bead ED 2 hr + TFA
Lane 7	Wash 10	Lane 17	Bead ED 2 hr w/o TFA
Lane 8	Wash 26	Lane 18	Bead ED 4 hr + TFA
Lane 9	Wash 51	Lane 19	Bead ED 4 hr w/o TFA
Lane 10	Std. HI	Lane 20	Low range Protein marker

Figure 6 describes the enzymatic digestion of HPI in presence of CPB, also the leachability of immobilized recTryp. In lane 1 a 14 kDa fragment of undigested HPI was observed which indicates that CPB does not act on HPI in the absence of recTryp. Absence of a 14 kDa fragment in lane 2 indicates that the Standard recTryp used for immobilization is active and it completely digested the HPI. Absence of a 14 kDa fragment in the lane 3 indicates that even after Biotinylation, recTryp retained its activity and it efficiently digested the HPI. In lane 4 a fragment slightly lower than 14 kDa was observed indicating that traces of recTryp were present in the 1st wash thus generating intermediates while digesting HPI. The single fragment observed in lanes 5-9 indicates undigested HPI. Hence there is possibility that residual unbound recTryp was efficiently removed in the later washes.

Lane 12-19 shows ED of HPI with immobilized recTryp indicating no digestion after 30 mins and 1 hour but lane 16-19 shows increased conversion of HPI to HI. Lane 17 and 19 shows similar pattern as seen in lane 16 and 18 indicating no leachability of recTryp from the matrix. The above statements confirm successful immobilization of recTryp on streptavidin coated magnetic particles.

DISCUSSION AND CONCLUSION

In the current study, an attempt was made to immobilize recTryp onto SCM beads for efficient enzymatic conversion of insulin. Biotinylation reduces the activity of recTryp due to binding of biotin moieties to the catalytic site of recTryp as the NHS activated groups attacks the 1^{o} amines in the proteins. Complex formation between streptavidin and biotinylated rec Tryp was initially assessed to confirm the immobilization. The activity of immobilized recTryp is difficult to monitor with the current activity assay, hence SDS-PAGE based assay was adapted which proved a reliable tool to assess the activity of immobilized enzymes. If executed critically, the SDS-PAGE assay can prove its ability to quantify the extent of immobilization with accuracy. The same can give an idea about leachability of immobilized enzyme. The immobilization method adapted can be implemented at commercial scale to stop the enzymatic digestion reaction effectively. In insulin generation, the controlled digestion of precursor protein to obtained target molecule is required due to usage of free proteolytic enzymes. This can be overcome by the usage of immobilized recTryp on magnetic beads as it facilitates the ease of separation of enzyme from the digestion reaction hence, the control over further digestion of target molecule is prevented which in turn will have effect on cost of insulin manufacturing process. Also, due to control over digestion, digesting enzyme can be used in excess to increase the rate of reaction. This will reduce the process time of insulin generation. The reusability of immobilized recTryp would prove an added advantage to reduce the process cost.

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