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PURIFICATION AND CHARACTERIZATION OF PEGYLATED FORM OF RECOMBINANT L-ASPARAGINASE II FROM *ESCHERICHIA COLI*

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ABSTRACT

The anti-leukemia, L-Asparagnase II (L-ASP) causes hypersensitivity reactions by forming neutralising antibodies. One of the approaches to minimise hypersensitivity of L-ASP is to modify biological and physicochemical properties without changing its activity. Thus, the present study was aimed to PEGylation of recombinant L-ASP from Escherichia coli. L-ASP was PEGylated and was purified on Q-Sepharose column. Purity of PEGylated L-Asp was found to be 100% with enzyme activity of 250± 3.4 IU. In conclusion, the PEGylation was successfully achieved with high pure enzyme.

KEY WORDS

L-Asparagnase, PEGylation, Escherichia coli, enzyme activity

INTRODUCTION

L-Asparaginase II (L-ASP) is an enzyme used to treat around 80% of acute lymphoblastic leukemia (ALL) cases. The mechanism of action of L-ASP is hydrolysing of L-asparagine and formation of L-aspartic acid and ammonia in cancer cells. Cancer cells unable to synthesize amino acid L-asparagine, thus L-ASP deprive this amino acid and lead to cell apoptosis [1]. For therapeutic purpose currently, the key sources of L-ASP are from *Escherichia coli* and *Erwinia chrysanthemi*. The main limitations of L-ASP from these sources in the therapeutic field include, low stability, incidence of hypersensitivity, and short half-life due to rapid clearance in the body [2]. The hypersensitivity reactions of L-ASP caused due to formation of neutralising antibodies and have clinical importance. For increasing half-life and decreasing the immunogenicity of L-ASP, great effects have been made [3]. One of the approaches to minimise hypersensitivity of L-ASP is to modify biological and physicochemical properties without changing its activity [4]. Polyethylene glycol (PEG) is used for covalent attachment with L-ASP to overcome of immunogenic reactions. PEGylation of L-ASP also helps to improve the circulation time in blood, resistance against proteolytic digestion in serum and increases the stability [5]. Previous researchers reported that the PEGylated L-ASP from *E.coli* shown decreased immunogenicity and increased circulating half-life [4, 6]. In this scenario, the present study was aimed to PEGylation of recombinant L-ASP from *E. coli* and its purification.

MATERIALS AND METHOD

PEGylation of L-ASP

The produced recombinant L-ASP from *E. coli* at Virchow Research Center, Hyderabad was used for the PEGylation in the current study. PEGylation was carried by adding 1:5 ratio of recombinant L-ASP (5mg/ml) in 50 mM phosphate buffer (pH 7.0) and mPEG 5KD Succinimidyl Succinate into reaction vessel under slow stirring at 2-8°C for 12 hours. The reaction was

quenched by 10mM glycine [4] and the PEGylated L-ASP was estimated by Lowry's method [7].

Purification of PEGylated L-ASP

PEGylated L-ASP was purified on Q-Sepharose ion exchange column [3]. Briefly, to the pre equilibrated column (50mM phosphate buffer, pH 7.0) the sample was loaded and followed by post load wash with equilibrate buffer. The protein was eluted with linear gradient of 0.5 M NaCl (42-55%) and the elution was subjected to dialysis against 10mM phosphate buffer (pH 7.0). The purified PEGylated protein was estimated by Lowry's method [7].

Purity analysis of PEGylated L-ASP

Purity analysis of enzyme was carried out by size exclusion chromatography (SEC) - HPLC equipped with TosoH TSKgel G3000SW column. The column was equilibrated with 50 mM Tris-HCl, 300 mM NaCl, pH 7.5 and 20µl of 2.0 mg/ml purified PEGylated L-ASP was injected in the column. Equilibration and elution was carried out at a flow rate of 1 ml/min. Gel filtration marker proteins (GE Healthcare, Sweden) were applied on the column for estimation of purity of PEGylated L-AS [8].

SDS-PAGE and MALDI-TOF analysis

SDS-PAGE and MALDI-TOF analysis were carried out for characterization of PEGylated L-ASP. SDS-PAGE analysis used to differentiate between free L-ASP and PEGylated L-ASP. Two independent SDS-PAGE gels were used, first gel was stained with titrisol for detection of free PEG and PEGylated L-ASP and the second gel was stained with commesieve brilliant blue. The images were obtained with gel dock and the results were investigated [9]. The purified PEGylated L-ASP was subjected to MALDI-TOF for molecular weight determination [10].

Determination of PEGylated L-ASP enzymatic activity

Nessler's reagent method was employed to determine the enzyme activity of PEGylated L-ASP [11]. To the 900 µl of buffer (50 mM Tris-HCl and 50 mM KCl, pH 8.0), 100 µl of PEGylated L-ASP in was added and incubated for 30min at 37°C. After incubation, 100 µl of 15% trichloroacetic acid was added to stop the reaction and mixture was centrifuged (10,000g, 4°C, 5 min). To the supernatant (100µl), 100µl Nessler's reagent was added and the volume was made up to 1ml. Later the reaction was mixed, incubated for 10min at 30°C and absorbance was measured at 436 nm. The produced anomia was quantified against standard curve with ammonium sulphate and the activity was expressed in IU.

STATISTICS

All experiments were carried out in triplicate and the results were expressed in ±standard error.

RESULTS AND DISCUSSION

L-ASP was used as a therapeutic agent for the curative of diseases ALL, acute mielomonocitic, Hodgkin's disease and melanosarcoma. Most commonly the L-ASP used in ALL, but undesired side effects such as pancreatitis, liver damage, renal complications. Apart from these, the L-ASP leads to hyper sensitivity reactions and has half-life and low stability [12]. To overcome above complications, PEGylation of L-ASP is the promising strategy. In the current study the recombinant L-ASP was PEGylated with m-PEG 5KD with 90± 2.1 % efficacy.

Earlier researchers purified PEGylated L-ASP by three or two step chromatography [3], in the current study we attempt to purify in single step on Q-Sepharose. The purified PEGylated L-ASP with yield of 79± 2.4 % and enzyme activity found to be 250± 3.4IU compared to crude (350± 2.4 IU). The crude PEGylated L-ASP showed high enzyme activity due to presence of free L-ASP and when purified the free L-ASP was removed in the column thus the purified enzyme activity was found to be low [13]. SDS-PAGE analysis was performed to evaluate the free L-ASP and PEGylated L-ASP, two different stains were used. For detection of PEG and PEGylated L-ASP titrisol and for normal L-ASP detection commesieve brilliant blue were used. The results showed in the purified PEGylated L-ASP, PEG was not detected in the gel stained with titrisol, indicated the efficiency of purification (Figure 1A-L4). Figure 1B showed the commesieve brilliant blue staining of PEGylated L-ASP and L-ASP. PEGylated L-ASP displayed a smear on gel with apparent masses indicating the random PEGylation**.** The protein concentration of crude and purified PEGylated L-ASP was quantified and was found to be 4.5± 0.5 and 2.3± 0.36 mg/ml respectively. The purified PEGylated enzyme was subjected to SEC-HPLC for the purity check and was found to be 100% purity and the results are in contrast with earlier studies [3, 4]. The MALDI-TOF analysis of PEGylated L-ASP results revealed that the molecular weight was found to be 341.5KD.

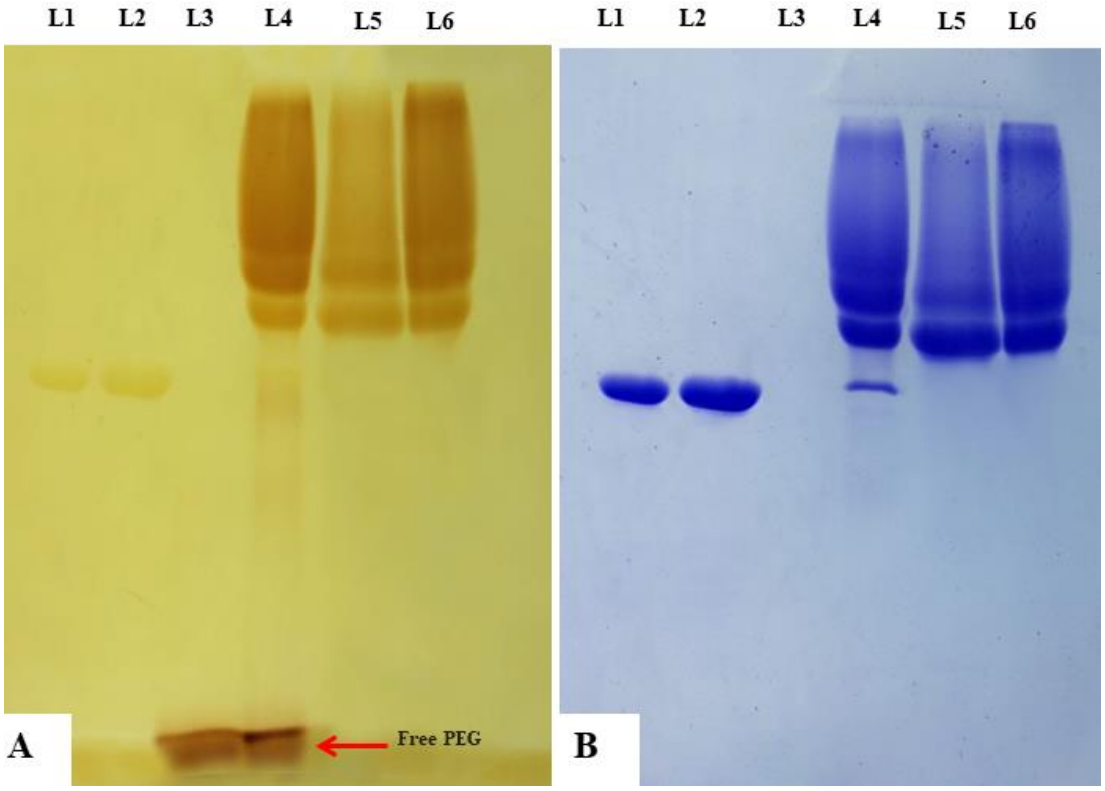
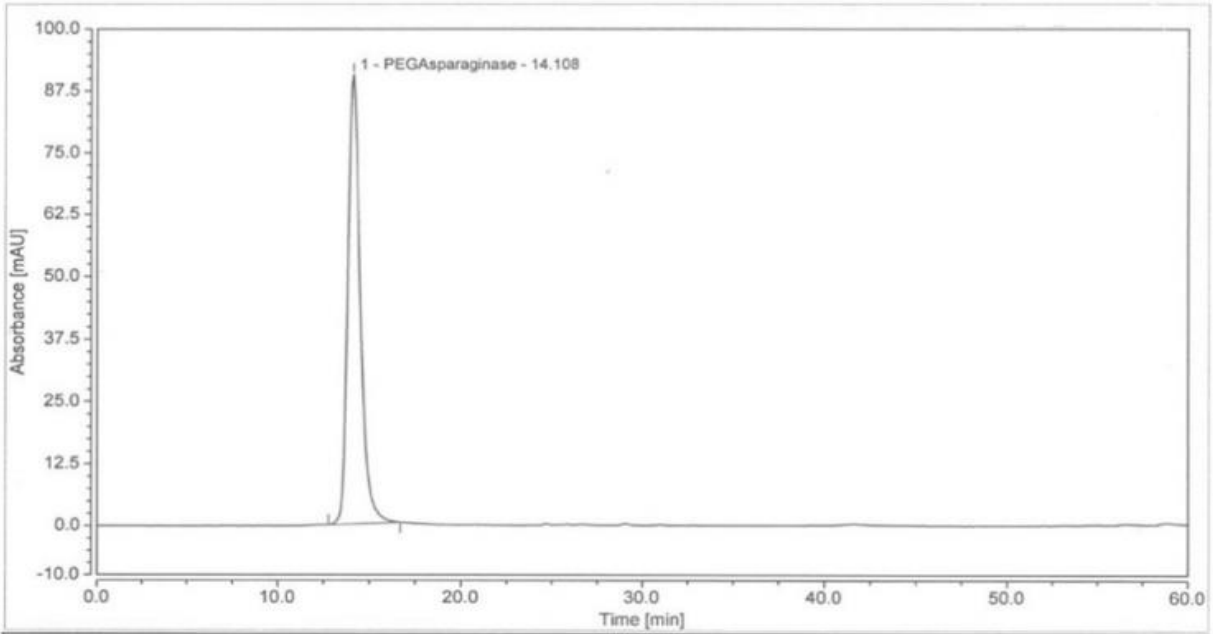


Figure 1A): Titrisol staining of SDS-PAGE gel; 1B): Commesieve brilliant blue staining of SDS-PAGE gel; Where; L1-Standard L-ASP, L2-Purified L-ASP, L3-m-PEG5KD, L4- PEGylated L-ASP, L5- Standard PEGylated L-ASP, L6-Purified PEGylated L-ASP



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	PEGAsparaginase	14.108	72.133	90.464	100.00	100.00	n.a.
Total:			**72.133**	**90.464**	**100.00**	**100.00**	

Figure 2: Purity analysis of PEGylated L-ASP on SEC-HPLC

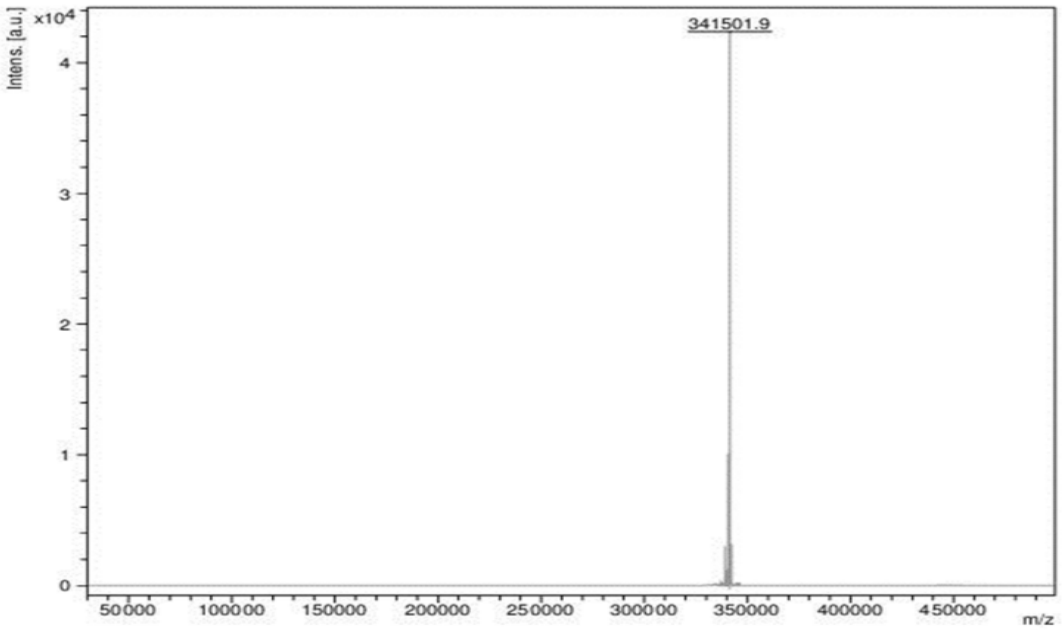


Figure 3: MOLDI-TOF analysis of PEGylated L-ASP

CONCLUSION

In conclusion, the PEGylation was successful with high purification efficiency. Thus, the current developed technology for PEGylation of L-ASP can be transferred to industry scale production. The further work will be Focused on clinical evaluation of PEGylated L-ASP.

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