INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 13, No. 3, p. 38-44, 2018

RESEARCH PAPER OPEN ACCESS

Cloning, expression and purification of L-asparginase II from *Escherichia coli* in *E. coli* $BL_{21}DE_3$

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Key words: L-Asparagnase II, *Escherichia coli*, Scale up, Sepharose, MALDI-TOF.

http://dx.doi.org/10.12692/ijb/13.3.38-44 Article published on September 07, 2018

Abstract

L-Asparagnase II (L-ASP) is a chemotherapeutic enzyme catalyses hydrolysis of asparagine into aspartate and ammonia, a key mechanism for tumour cells. L-ASP widely used for the treatment of acute lymphoblastic leukaemia and has commercial value. In this scenario, present study was aimed to sale up of recombinant L-ASP from *Escherichia coli*. L-ASP gene was cloned in pET 21a vector and was expressed in *E. coli* $BL_{21}DE_3$.The pH (7.2) and temperature (37°C) were optimized in shake flask, were maintained during scale up (3L and 30L) and the expression was recorded. Expressed L-ASP was captured by Diethyl amino ethyl sepharose, polished on quaternary ammonium sepharoseion exchange chromatography and purity was found to be 99.24%. The estimated yield calculated to be 1.5gm/Land the enzyme assay of purified enzyme was assayed to be 450 IU. Molecular weight of L-ASP monomer was determined by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and L-ASP tetramer was estimated to be 138.92kilo Daltons. The present study concluding that the developed process might throw insights in the scale up of at industrial level for high production.

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Introduction

L – asparaginase II (L-ASP) is an enzyme, widely used as drug for acute lymphoblastic leukaemia (ALL) (Aghaiypour *et al.,* 2001). L-ASP plays a major role in the cell proliferation, but in the cancer cells the L-ASP can't produce due to lack asparagine synthatase. Thus, the L-ASP is an attractive target as a chemotheraptic drug for cancer cells with no harm to normal cells. In cancer cells, L-ASP inhibits the protein synthesis by L-asparagine hydrolysis and degrades the L-asparagine amino acid thus leads to cell death (Ebrahiminezhad *et al.,* 2011).

L-ASP is commonly present in microorganism, plants and animals. Among all, the better source for L-ASP is from microbes because they can be cultured easily, cost-effective and eco-friendly in nature (Jayam and Kannan, 2014). For the treatment ALL, clinically approved sources were from *Escherichia coli* and *Erwinia chrysanthemi* (Avramis and Panosyan, 2005).Besides the use in health care, L-ASP gain attention in the food processing industry for destruction of acrylamide, a neurotoxin especially. Apart from these, asparagine biosensors have been developed for detection of measuring of asparagine in normal and leukemia serum samples by entrapping the L-ASP in between on dialysis membranes (Aghaeepoor *et al.,* 2011). As L-ASP has industrial value, the scale up is needed in cost effective manner. In this scenario, the aim of the present study was cloning, expression, scale up and purification of recombinant L-ASP from *E. coli.*

Materials and methods

Cloning of L-ASP

For cloning and expression, the *E. coli* was grown for 8h,at 37°C and the DNA was isolated. L-ASP gene was amplified, enzyme digestion and was cloned in to pET 21a vector (Sambrook *et al.,* 1989) with selected ampicillin resistant markers gene. The cloning was confirmed by the colony PCR and restriction digestion with Hho1 and Nde1 followed by agarose gel electrophoresis. The cloned vector was initially transformed in to *E. coli* DH5α cells and the cells were grown in LB medium with 100 µg/mL ampicillin. The positive clones were chosen for plasmid isolation and the isolated plasmids were transformed to *E. coli* $BL_{21}DE_3$ competent expression cell. Later the L-ASP expression was recorded and the research cell bank was prepared for further studies.

Pilot study and scale up of L-ASP

Initially the clones were fermented in 300mL shake flask and 3L fermenter in LB broth. The fermented conditions were optimised with pH (7.2) and temperature (37°C) with addition of 100µg/mLampicillin. The rpm was maintained at 180 and induced with1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG)after 6 h of growth (Kishore *et al.,* 2015). The expression was analysed on 12% SDS- PAGE (Raymond and Weintraub, 1959). For scale up in 30 L, the terrific broth was used as medium along with trace elements as describe earlier, and fed batch fermentation technique was used (Mitra *et al.,* 2017). Briefly, the fermenter was cleaned and medium was sterilized. The cloned L-ASP in *E. coli* $BL_{21}DE_3$ seeded in to the medium and the temperature (37°C), pH (7.2) was maintained. Once the OD of the culture was reached 60, the induction was done with 1mM IPTG and the cells were harvested at OD - 70. Initially the rpm was maintained 200 and increased with cell mass up to 500 up until the batch harvesting.

Purification of L-ASP

Harvested cells were centrifuged at 4,000 rpm, 4°C for 30min. The pellet was suspended in extraction buffer and the cells were lysed with high pressure homogeniser at 900 bar for 3 cycles. The lysed sample was centrifuged at 14,000 g at 4°C for 30min. The supernatant was to filtration through 0.45 µm filtration cassette and then subjected to column purification. Initially the L-ASP was captured on pre-equilibrated (50 mM Tris, pH-8.6) Diethyl amino ethyl cellulose (DEAE) Sepharose and eluted with 50 mM Tris and 150mM NaCl, pH-8.6. Later the captured protein was concentrated and buffer exchange byultra-diafiltration. The concentrated protein was then polished on quaternary ammonium (Q)-Sepharose equilibrated with 25mM phosphate

buffer, pH-7.4, eluted with 100NaCl (Gladilina *et al.,* 2009). The purity was checked by SDS-PAGE, SEC-HPLC and the molecular weight was determined by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF).

L-ASP enzyme assay

Enzymatic activity was assessed by Nessler's reagent method (Zuo *et al.,* 2014). Briefly, 100 µL of L-ASP in was incubated with 900 µL (50 mM Tris-HCl buffer, 50 mM KCl, pH 8.0) for 30min at 37°C. After incubation, the reaction was stopped by adding 100 µL of 15% trichloroacetic acid and the mixture was centrifuged (10,000g, 4°C, 5 min). To the 100 µL supernatant, 100µL Nessler's reagent and 800µL distilled water was added. The reaction mixture was vortexed, incubated for 10min and the absorbance was measured at 425 nm. The produced anomia was quantified against standard curve with ammonium sulphate and the activity was expressed in IU.

Results and discussion

In the present study, we produced recombinant L-ASP by cloning of L-ASP gene from *E. coli*, into pET 21a vector and transformed to *E. coli* $BL_{21}DE_3$ expression system (Fig. 1). *E. coli* was grown in LB medium was used for isolation of DNA, isolated DNA was checked for its purity and was found to be good quality.

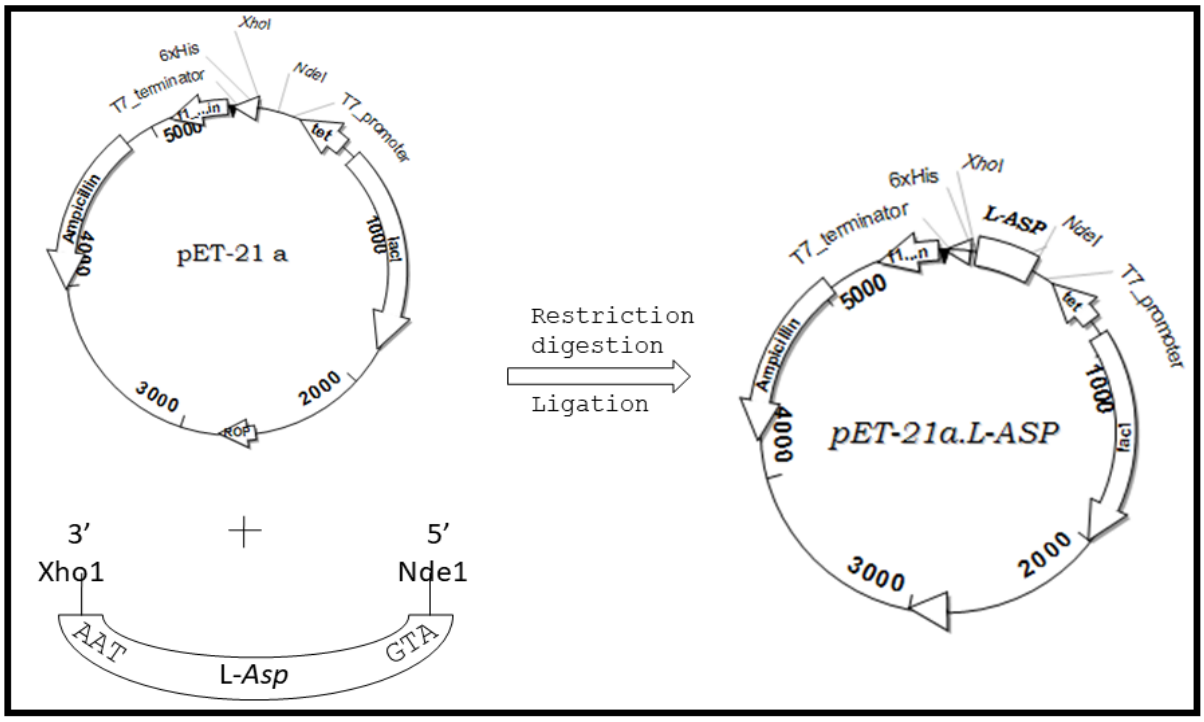


Fig. 1. Construction and cloning of L-ASP gene in pET 21a vector.

The isolated DNA was amplified by PCR and was cloned in to pET 21a vector with 80% cloning efficiency (Fig. 2). Colony PCR of L-ASP gene also confirmed the cloning of L-ASP in vector. Later the vector was digested with Hho1 and Nde1 and the digested product were run on agarose gel electrophoresis, conformed successful cloning (Fig. 3). Further the clone was transformed in to *E. coli* DH5α and the plasmid was isolated and transformed into expression vector, *E. coli* $BL_{21}DE_3$. For pilot study (300ml shake flask) the pH and temperature were standardized and found to be 7.4 and 37°C respectively and the expression was recorded. After recording the expression in 300ml, the scale up studies with 3L and 30L fermenter were carried. For scale up process the optimisation of pH and temperature plays a key role and should maintain constantly (Yong *et al.,* 2018). In the current study, the pH and temperature have been maintained constantly.

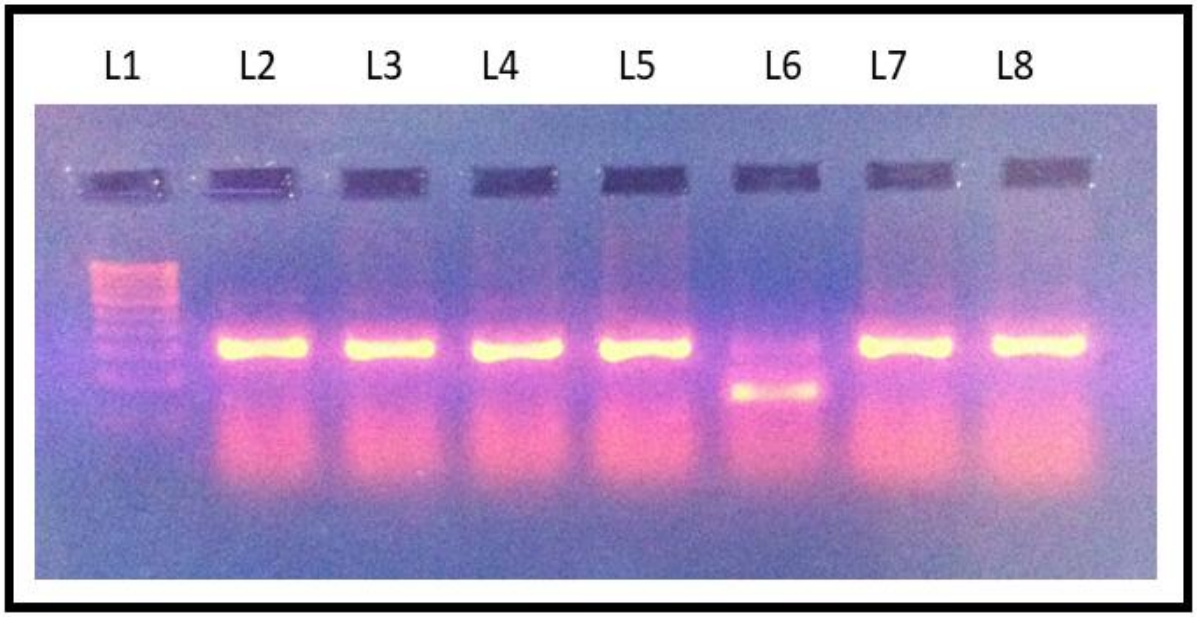


Fig.2. Putative recombinants' identification by colony PCR.
Where, L1- DNA Ladder; L2 8 - Transforments,

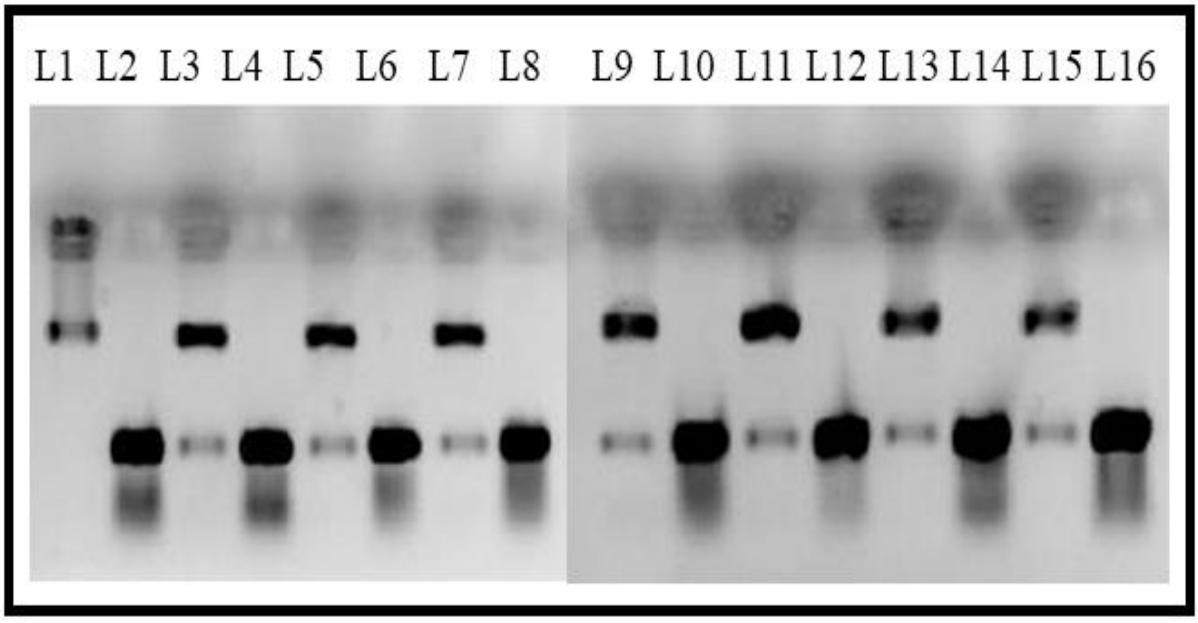


Fig. 3. L-Asp release from Cloned Vector.
Where, L1-Linearized vectop ET 21a; L2- PCR product of L-ASP; L- 3,5,7,9,11,13,15 - restriction digested positive clone vector; L4,6,8,10,12,14,16 – PCR product of L-ASP amplified from positive clone.

The fed batch fermentation (30L) was performed at optimized conditions and the harvested batch was lysed and centrifuged. Fig.4 showed the expression pattern of L-ASP in $BL_{21}DE_3$in compared with standard L-ASP. The crude protein was analysed for its enzymatic activity and the enzyme activity was calculated to be 100 IU. Krasotkina *et al.,* (2004) purified recombinant L-ASP by one step chromatography, in the current study L-ASP was purified in two steps. Initially the crude enzyme was captured on DEAE cellulose and the purified protein yield showed30%. The purity of the protein calculated upon subjected to SEC-HPLC was found to be 90% and the enzyme activity was found to be300 IU Later the DEAE purified sample was subjected to ultra-diafiltration.

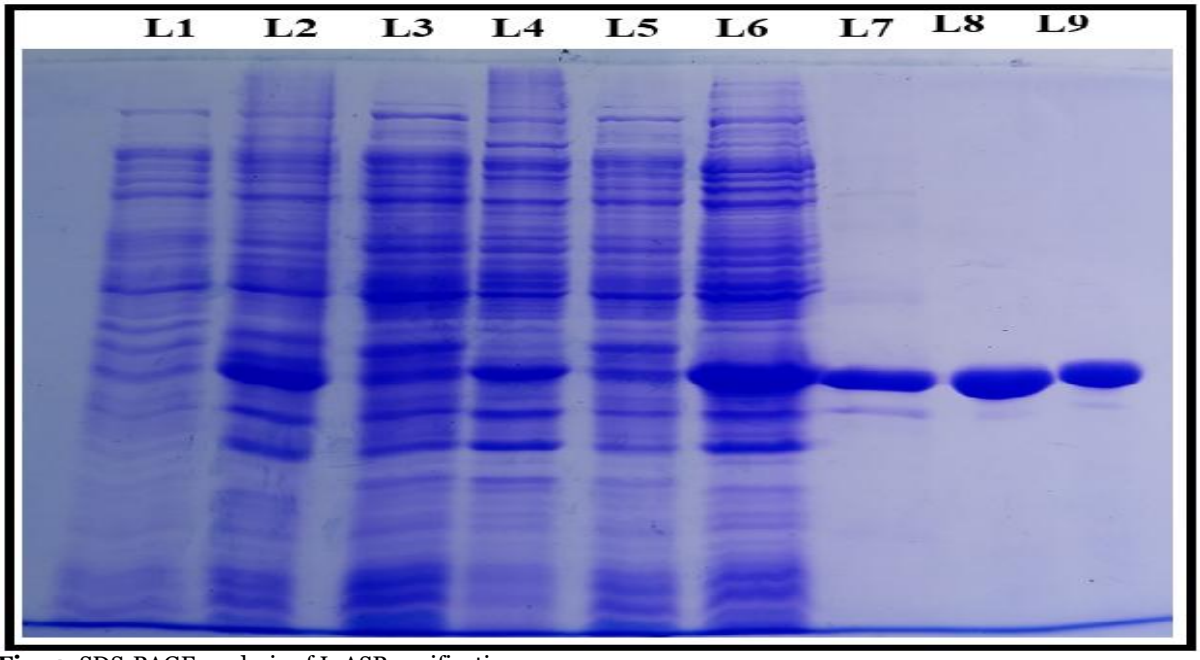
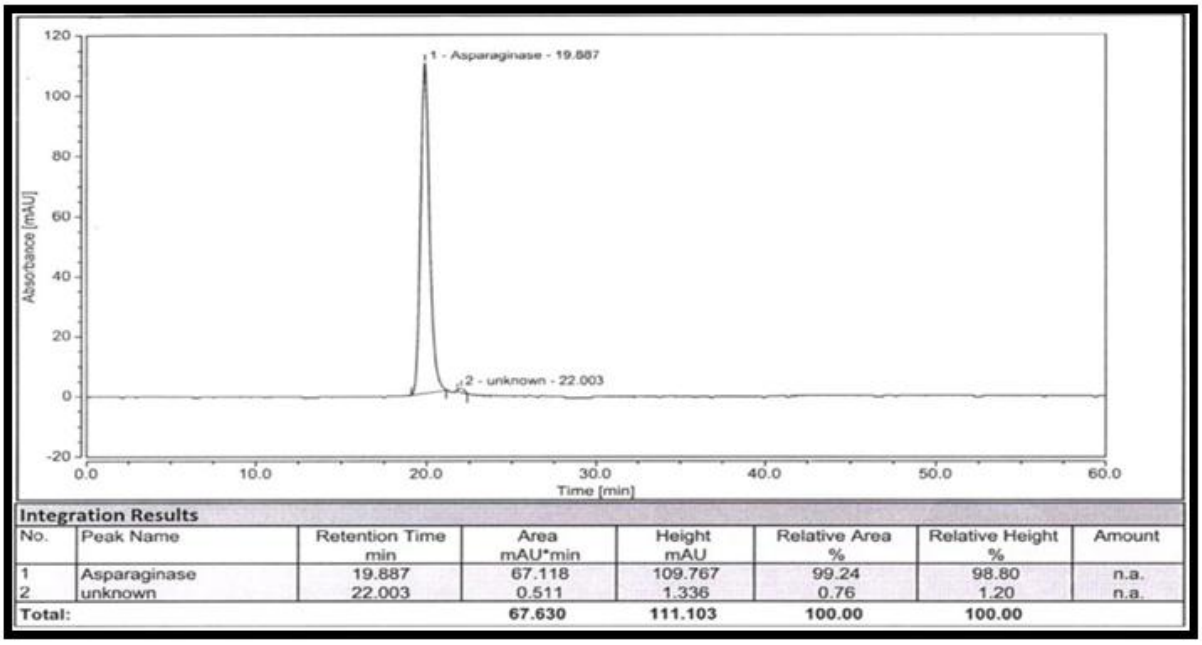


Fig. 4. SDS-PAGE analysis of L-ASP purification.

Where, L1-Shake flask un-induced; L2- Induced harvest; L3- 3L un-induced; L4- - Induced harvest; L5-30L un-induced; L6- Induced harvest; L7- DEAE capturing; L8 Q-Sepharose polishing; L9-Standard L-ASP.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	Asparaginase	19.887	67.118	109.767	99.24	98.80	n.a.
2	unknown	22.003	0.511	1.336	0.76	1.20	n.a.
Total:			**67.630**	**111.103**	**100.00**	**100.00**	

Fig. 5. SEC HPLC analysis of purified L-ASP.

The ultra-diafiltrate was polished on Q-Sepharose and the purity was found to be 99.24% on SEC-HPLC. Fig.3. showed the purification pattern of L-ASP. The purified protein was estimated and yield was found to be 1.5gm/L with percentage yield of 20%. 12% SDS-PAGE analysis showed single and prominent band confirms the purity of L-ASP (Fig. 5). When purified L-ASP was studied for its enzymatic activity, the activity was showed 450 IU maximally this result is in contrast with earlier findings (Cammack *et al.,* 1972; Pourhossein and Lee *et al.,* 1989; Korbekandi, 2014). MALDI-TOF determines the molecular weight (34.73) of L-ASP monomer and tetramer was found to be estimated to be 138.92 KDA (Fig. 6) and the result is in accordance with preceding studies (Kishore *et al.,* 2015).

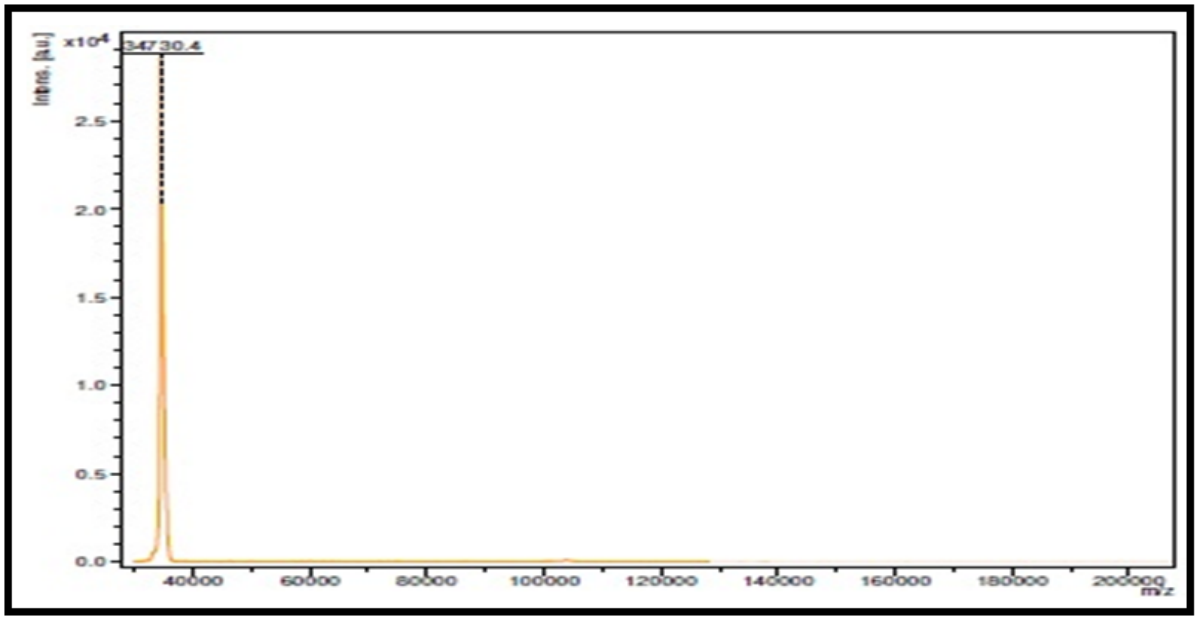


Fig. 6. MALDI-TOF analysis of purified monomer of L-ASP.

Conclusion

The current study established optimized production of L-ASP from 300 mL to 30L scale and also shown the consistent expression of L-ASP throughout the scale up. The purification optimization showed the high purification and specific activity. Thus, the current developed technology for scale up of L-ASP can be translated to industry scale production. L-ASP having allergic side effects, thus further studies will be focused on PEGylation of L-ASP at industrial scale and its preclinical evaluation.

Acknowledgement

The authors are grateful to the Virchow Research Center, Hyderabad for their support to carry out the research work.

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