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Original Article

CHARACTERIZATION OF URATE OXIDASE EXPRESSED IN *ESCHERICHIA COLI*

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ABSTRACT

The biological market is increasing day by day, so the regulatory bodies are becoming more stringent to maintain the quality of the product. To meet the highest quality standards, it is necessary to assess the quality of the product, in each and every step of the bio-therapeutic drug manufacturing. Originator molecule manufactures usually define their own standards, but where as a biosimilar producer should do head to head comparison with the standard molecule, in order to prove it as closely similar to the standard with respect to structure and function.

In this paper, we compared urate oxidase produced in *E. coli* with the marketing Elitek which is expressed in yeast. We used various methods to determine its molecular weight, structural integrity, *In-vitro* bioassay and other methods to compare with the standard. Integrity by DSS cross linking is first of its kind for urate oxidase, expressed in E.coli.

Keywords: Analytical methods, Uricase, SDS-PAGE, RP-HPLC, and DSS cross linking.

INTRODUCTION

The present biotechnology industry usually employs bacteria, yeast, insect cells or mammalian cells for the production of high value therapeutic proteins. These products are complex in structure, size and biological activity. The path of a biotechnology product manufacture is cloning, cell banking, fermentation, purification and formulation. The quality and safety of the end product depends on the in-process. A minor change in the process leads to major changes in the quality of the product characteristics. So the quality assessment of the product has to be carried throughout the process starting from cloning to product release.

Thus, Analytical methods play a crucial role in the process development and production of a product. Therapeutic proteins are life saving products, thus the purity, identity, stability and performance of the product is of great importance.

Regulatory bodies have defined the limit of impurities, identity and quality of the product. It is necessary to meet the defined specification by the regulatory bodies to move the product into the market. So, Quality control plays a major role to market a manufactured product. Uricase (Urate oxidase) is human therapeutic protein used to treat Hyperuricemia [1] and tumor lysis syndrome[2], which are associated with the chemotherapeutic treament of cancer.In this research paper, we used various analytical tests to analyse the structural, physico-chemical and biological properties of urate oxidase showing its comparability with the originator molecule.

MATERIALS AND METHODS

Chromatography sorbents and columns were purchased from GE life sciences, chemicals were purchased from Sigma Aldrich, Iso-Electric Focusing pI marker were purchased from Biorad, glassware and other chemicals were of analytical grade purchased from local companies. Urate oxidase from *Aspergilus flavus* was cloned in *E.coli* [3] and then, the protein was purified using various chromatographic methods (submitted for publication).The purified protein was used for the study.

Molecular mass determination by SDS-PAGE

The molecular weight of uricase was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as per the method described by Laemmli [4]. The analysis was carried out using 12% resolution gel. The protein bands were detected by silver staining method [5].

Determination of pI by Iso-electric focusing (IEF)

The pI of the protein is determined by co-running the protein of interest and the ready to use pI markers in an electrophoresis. By exploiting the position of the known pI protein band, the pI of the Uricase can be determined [6].The protocol engaged is according to the Biorad's IEF instruction manual [7].

Identity of urate oxidase by western blotting

Purified uricase was loaded in the gel and allowed to run until the blue dye entered into the gel front and the bands were transferred to the PVDF membrane by semi-dry blot apparatus (Bio-Rad) for 40 min at 10 volts. The transferred membrane was blocked with 3% BSA in 1X PBS and incubated for overnight at 4^0C with primary antibodies raised against the Rasburicase in mouse (Data not shown). Then the membrane was washed thrice with 1X PBS supplemented with 0.1 % Tween, later the membrane was incubated for 2 hours in 1X PBS supplemented with secondary antibody conjugated with Alkaline phosphatase. Then the membrane was washed thrice with 1X PBS supplemented with 0.1 % Tween. Finally, the membrane was developed by NBT/ BCIP [8,9].

Determination of purity by reverse phase- high performance liquid chromatography (RP-HPLC)

10 µg of purified sample was loaded onto to the Chromosil C18 column (250 mm X 4.6 mm, 5 µm) which was pre equilibrated with 0.08% trifluoro acetic acid (TFA) in WFI and eluted by using 0.08% TFA in solvent with acetonitrile/water (6:4 v/v ratio) with a run time of 35 minutes [10]. The purity of the peak was analysed.

Determination of homo-tetramer confirmation by chemical cross linking of uricase with Disuccinimidyl suberate (DSS)

In order to determine the integrity of the uricase as homo-tetramer, uricase was cross linked with the DSS, which usually reacts and links the molecule which is near to the 10^0A vicinity [11,12,13,14].0.1and 0.2 nmol of purified uricase was cross-linked by incubating with 0.15 mM DSS for 30 min at 25^0C. The reaction was stopped by adding glycine to a final concentration of 50 mM. The cross-linked proteins were analysed by on 12% SDS-PAGE gel under reducing conditions, and the gels were developed by silver staining [5].

Determination of *In-vitro* activity of Uricase

In-vitro activity assay was carried out in 0.1 M sodium borate buffer (pH8.6) at 25°C, according to the method described by Mahler. [15]. The decrease in the uric acid concentration in the sample during the

enzymatic reaction was measured at 293nm.One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of uric acid per min at 37°C.

RESULTS AND DISCUSSION

The basic properties of Uricase were shown in the Table 1.

Table 1: It shows the basic properties of Uricase

Basic features of Uricase	1.	pH stability of uricase – 8.0
	2.	Optimum temperature - 25-37^{0}C
	3.	pI of the protein - 7.5- 8.0
	4.	Molecular weight of monomer - 34.0 kDa
	5.	Molecular weight of Tetramer - 136 kDa

Molecular mass determination by SDS-PAGE

SDS-PAGE is tool to identify the molecular mass of the protein, the identity of the protein and visualize the impurities in the gel, if any present. Determination of the molecular weight of uricase is an important method to identify the protein and to confirm the primary structure. Molecular weight of purified urate oxidase was determined as around 34.0 kDa by SDS-PAGE analysis (Figure 1).

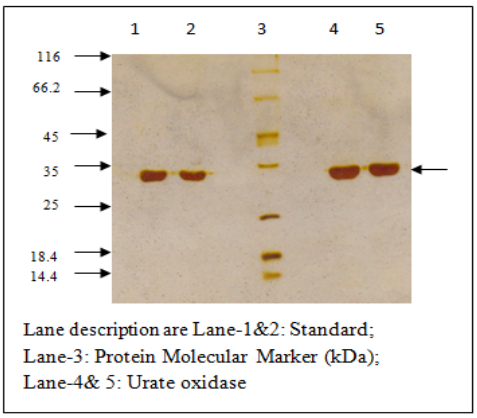

Fig. 1: It shows the determination of molecular weight of Uricase by SDS-PAGE

Determination of pI by Iso-Electric Focusing (IEF)

The change in the pI of the protein leads to the instability of the product and also loss of activity of the protein. Hence, it is essential to determine the pI of the protein during the process development, manufacturing, and the drug product. The pI of the protein is determined by IEF. The pI of the purified urate oxidase is around 7.5-8.0 (Figure 2).

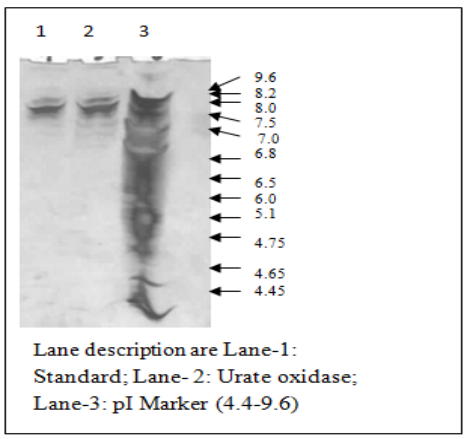

Fig. 2: It shows the pI of purified recombinant urate oxidase using IEF

Identity of urate oxidase by Western Blotting

Identity of the sample was confirmed by western blotting. Western blot analysis showed that the induction band confirmed the uricase (Figure 3).

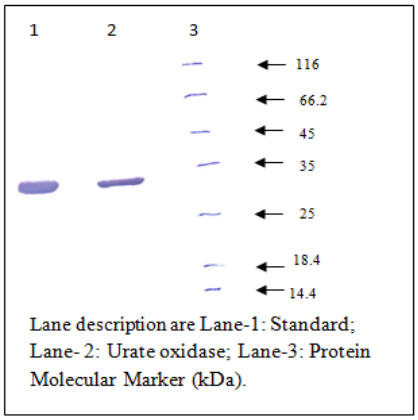

Fig. 3: It shows the western blot of purified recombinant urate oxidase

Determination of purity by RP-HPLC

Purity of the sample was determined by RP-HPLC with C18 column, revealed that uricase was eluted at 27.8 min and more than 99.0 % pure (Figure 4).

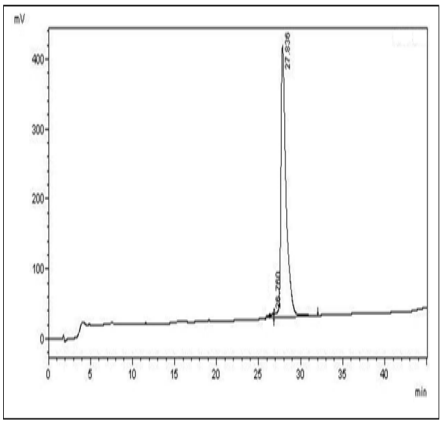

Fig. 4: It shows the RP-HPLC of purified recombinant urate oxidase

Determination of homo-tetramer confirmation by chemical cross linking of uricase with Disuccinimidyl suberate (DSS)

Urate oxidase has four monomers. The protein is unstable in the monomeric form and the stability is increased in the tetrameric form through the oligomeric association [16]. During the oligomeric association of the tetramers, they exhibit three different types of interface between the monomers. The active site of these monomers

is located at the interface [17].Integrity of the sample was determined by cross linking with the DSS. DSS cross linking of uricase confirmed the homo-tetrameric confirmation of the molecule (Figure 5).

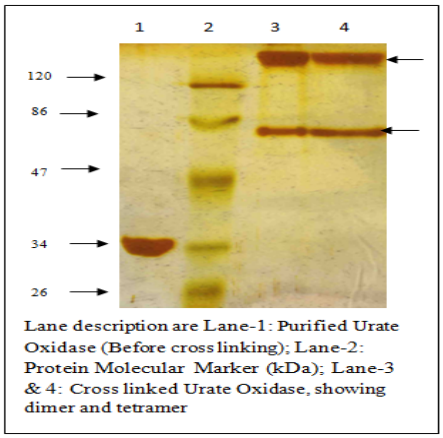

Fig. 5: It shows the DSS cross linking of purified recombinant urate oxidase for the determination of homo-tetramer

Determination of *In-vitro* activity of Uricase

In-vitro activity determination of Uricase was carried out and the results showed the substrate enzymatic utilization (Figure 6).The specific activity of the purified urate oxidase is 18.2 enzyme units per mg of protein, which is similar to the standard. As the purified protein shows similar activity to the standard, the integrity of the protein is also addressed [16].

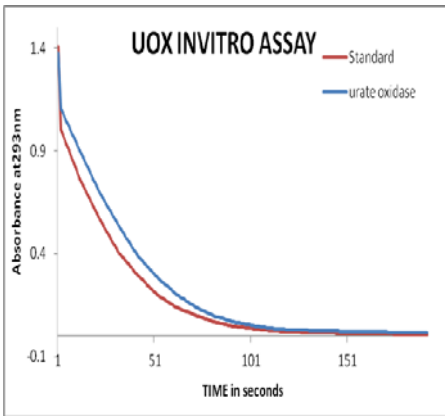

Fig. 6: *In-vitro* activity determination of purified recombinant Urate oxidase

CONCLUSION

Various analytical methods to characterise the purified protein are addressed in this paper. The molecular weight of the purified urate oxidase was around 34.0 kDa by SDS-PAGE analysis. Identity of the protein was confirmed by western blotting. Isoform distribution of the protein was acknowledged by IEF. Potency and purity of the product was analyzed by *In-vitro* assay and RP- HPLC respectively. The activity of the protein was determined by the enzymatic degradation of uric acid. Homo-tetrameric integrity of the sample was determined by using DSS cross linking, which is first of its kind for urate oxidase, expressed in E.coli.

ABBREVIATIONS

Iso-Electric Focusing (IEF), Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and Disuccinimidyl suberate **(**DSS) cross linking

CONFLICT OF INTERESTS

Declared None

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