

PURIFICATION AND EVIDENCE FOR THE INVOLVEMENT OF TRYPTOPHAN RESIDUE AT THE CATALYTIC SITE OF β -FRUCTOFURANOSIDASE FROM A THERMOTOLERANT YEAST *KLUYVEROMYCES MARXIANUS* NCYC 2675

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ABSTRACT

β Fructofuranosidase from *Kluyveromyces. marxianus* NCYC 2675 was purified using hydrophobic interaction chromatography. The purified enzyme had specific activity of 2116 μ moles/min/ml. Molecular weight was determined by gel filtration and SDS-PAGE. This was further confirmed by MALDI-TOF. The protein was found to be a non-glycosylated monomer of molecular mass 67 kDa and exhibited a pI of 3.7. Effects of amino acid specific modifying agents were studied to determine the amino acid residue at the catalytic site. NBS and HNBBBr, chemical modifiers of tryptophan showed concentration and time dependant linear inactivation of the enzyme. Modification with 60 μ M of NBS and 40 mM of HNBBBr resulted in 75% and 70% inactivation respectively. Substrate sucrose, and the products glucose and fructose, significantly lowered the extent of inactivation by the tryptophan modifying reagents. The reactions followed pseudo first-order kinetics and the inactivation kinetics indicated the presence of a single tryptophan residue near or at the catalytic site.

Keywords: β Fructofuranosidase, chemical modification, Tryptophan, purification, SDS-PAGE, *K.marxianus*.

INTRODUCTION

The genus *Kluyveromyces* represents one of the most extensively studied inulinase producers (Wang et al. 2000; Guerro et al. 2006; Wan et al. 2003). There are also reports of *Kluyveromyces marxianus* species producing high levels of inulinase EC 3.2.1.7 but there are only a few reports on invertase [β Fructofuranosidase EC 3.2.1.26] production from the same (Kushi et al. 2000; OngenBaysal and Sukan 1996; Rouwenhorst et al. 1990). In contrast to the limited data available for β Fructofuranosidase from *K. marxianus*, there are several reports on purification and extensive characterization from other yeast sources (Moreno et al.1990; Tanaka et al. 1998). To exploit new industrial potentials of

invertase, it is necessary to investigate new microbial strains and to understand the structure-stability relationship of this industrially and pharmaceutically important enzyme. The organism *K. marxianus* NCYC 2675, a thermotolerant yeast was therefore selected for enzyme production required for studies on some basic and applied aspects of this enzyme. Scanty information is available in the literature on the reaction mechanism and the active centers of invertase (β Fructofuranosidase) from *K. marxianus*. Leskovac have shown the role of tryptophanyl residues in catalysis of a commercial yeast β Fructofuranosidase (Leskovac et al.1975). Complete inhibition of invertase correlates with the oxidation

of 5.5 tryptophans per molecule of enzyme. Little else is known regarding the active site residues. Another report by Pons had indicated a direct implication of acidic residues in the glycoside hydrolase families in substrate binding and catalysis (Pons et al. 2003).

Reddy and Maley have proposed a mechanism for the hydrolysis of sucrose which involved Aspartate 23 as a nucleophile and Glutamate 204 as an acid / base catalyst through the procedure of affinity labeling and site-directed mutagenesis (Reddy and Maley 1996). Role of cysteine and the kinetic analysis has been studied by Waheed and Shall (1971). However no reports are available on the active site residues of β Fructofuranosidase, particularly from *K. marxianus*. In the present work, chemical modification studies were carried out to probe essential amino acid residue in β Fructofuranosidase from *K. marxianus* NCYC 2675.

MATERIALS AND METHODS

Materials

Sucrose, glucose and fructose were obtained from HIMEDIA, India. HNBBBr (2-hydroxy-5-nitrobenzyl bromide), was obtained from ICN Biochemicals USA, NBS (N bromosuccinimide) was procured from SRL India. Octyl Sepharose CL-4B and Gel filtration molecular weight markers were obtained from Sigma USA. SDS-PAGE molecular weight markers were obtained from Bangalore Genei INDIA. 30 kDa centricons were obtained from Millipore Corporation, UK. All buffers were prepared in glass distilled water.

All other chemicals used were commercially available high purity or analytical grade compounds.

Methods

Purification of the enzyme

Kluyveromyces. marxianus NCYC 2675 cells were grown in MSYP medium and harvested after 48 hours of growth by centrifugation at 6000 rpm for 30 minutes in a Sorvall RC-5B refrigerated centrifuge at 10°C. Freshly harvested cells were washed with 50 mM acetate buffer, pH 4.5 and kept at 4°C till further required.

Batches of about 25g packed cells (obtained from 1 liter medium) were worked up at a time. Cells were suspended in 50 mM acetate buffer and homogenized using Braun's homogenizer. The clear supernatant which contained the activity was processed further. It was subjected to fractional precipitation by the slow addition of finely ground ammonium sulphate under stirring on ice. The fraction which precipitated at 40% saturation was removed by centrifugation at 10,000 rpm for 30 minutes, checked for activity, and discarded. The remaining supernatant showed activity and was loaded on octyl column 2.0 cm X 10.5 cm pre-equilibrated with 50 mM acetate buffer, pH 4.5 containing 20% ammonium sulphate. The supernatant (containing crude enzyme) was treated with solid ammonium sulphate to a final concentration of 20%w/v and loaded on the octyl column. One bed volume buffer (containing 20% ammonium sulphate) wash was given to remove the unbound protein. This was followed by elution with 50 mM acetate buffer pH 4.5. The flow rate of the column was maintained at 1.5ml/min and fractions of 3.0 ml quantity were collected. The enzyme eluted between 50-60 ml and the fractions showing enzyme activity were pooled (approximately 12 ml).

Pooled fractions showing activity in the previous run were loaded on a successive octyl column washed and equilibrated as described above. The enzyme eluted in fractions between 40-46 ml. The fractions were concentrated using 30 kDa centricons at 4°C and later by lyophilization using speedvac SVC 100. Protein and enzyme activity was determined and Native and SDS-PAGE was performed using 7.5% gels to check the homogeneity of the enzyme. Protein bands were visualized by silver staining and Commassie blue staining.

The purified enzyme was stored at 4°C till further use.

Enzyme Assay

Activity measurements were done using DNSA by the spectrophotometric method (Miller 1959). Protein was estimated using bovine serum albumin as a standard (Lowry et al. 1951).

Determination of the carbohydrate content

The carbohydrate content of β Fructofuranosidase from *K. marxianus* NCYC 2675 was determined by phenol-sulphuric acid method (Dubois et al.1956).

Enzyme solution (750 µg protein measured by Lowry method) was diluted to 200 µl using distilled water. 200 µl of 5% phenol reagent was added and the mixture was kept for 10 minutes at 28°C, followed by the addition of 200 µl of concentrated sulphuric acid. The mixture was cooled to room temperature before reading the absorbance at 490 nm. The carbohydrate content was calculated using a standard curve prepared with mannose (10µg-100µg/ml)

Isoelectric focusing

Isoelectric focusing in polyacrylamide gel was performed (Vesterberg 1972). The tube gels were prepared in duplicate of the following composition: 835 µl of 30% acrylamide, 125 µl ampholine, 150 µl of 10% APS, glycerol 290 µl.

Molecular Weight Determination

A) Gel Filtration

Molecular weight of native enzyme was carried out by gel filtration on a Sephadex G-200 column (Andrews 1965). M_r the relative molecular weight was determined by gel filtration chromatography using protein standards under identical conditions (Sigma MW-GF-200Kit) which include β amylase (M_r 200 kDa), alcohol dehydrogenase (M_r 150 kDa), bovine serum albumin (M_r 66 kDa), carbonic anhydrase (M_r 29 kDa) and cytochrome c (M_r 12 kDa).

B) SDS-PAGE.

Samples were analyzed on native polyacrylamide gel electrophoresis to check the purity and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) to determine the molecular mass of the purified enzyme using lower range molecular weight markers kit from Amersham Biosciences (Lammeli 1970). The kit marker proteins comprised of phosphorylase b (97kDa), bovine serum albumin (66kDa), ovalalbumin (43kDa), carbonic anhydrase (29kDa), soybean trypsin inhibitor (20kDa) and lysozyme (14.3kDa).

C) MALDI-TOF (Matrix assisted laser desorption/ionization time of flight mass spectrometry)

Mass spectral analysis was performed on a Voyager-De-STR (Applied Biosystems) MALDI-TOF. A

nitrogen laser (337nm) was used for desorption and ionization. Spectra were acquired in the range of 10 to 100 kDa, in linear mode with delayed ion extraction and with an accelerating voltage of 25 kV. The low mass ion gate was set at 4500 Da. The analysis was performed in four replications. The instrument was calibrated with myoglobin and bovine serum albumin.

5 µl of enzyme (500µg/ml) was mixed with 25 µl of Sinapinic acid (10mg/ml) prepared in 30% Acetonitrile (ACN) containing 0.2% Trifluoroacetic acid (TFA), by the dried-droplet method.

Chemical Modification

Preliminary experiments to test the effects of some amino acid specific reagents on β Fructofuranosidase from *K. marxianus* NCYC 2675 were performed (Means and Feeney1971). NBS and HNBBBr were used as modifying reagents for tryptophan. Purified β-D Fructofuranosidase(150µg) from *K. marxianus* NCYC 2675 was incubated at 28°C with different concentrations of NBS (10µM-60µM) in 50 mM sodium acetate buffer pH 4.5, or HNBBBr (10mM-40mM) in 50 mM sodium acetate buffer at pH 4.5 from freshly prepared stock solutions of NBS(1mM) and HNBBBr (500mM). Aliquots were withdrawn at regular intervals of time for the assay of enzymatic activity. Enzyme incubated in the buffer in absence of the modifying reagents served as control.

The titration of accessible tryptophan residues in β Fructofuranosidase with NBS was followed spectrophotometrically at 280 nm. The control cuvette had 50 mM sodium acetate buffer pH 4.5 and the experimental had β Fructofuranosidase (500 ug protein) in a total volume of 1ml of 50 mM sodium acetate buffer. Successive 10 µl aliquots of 20 µM NBS were added to both control and experimental cuvettes at 2 minutes interval and changes in absorbance at 280 nm was recorded till no further change in absorbance was observed. After each addition, an aliquot of 10 µl was removed and the reaction arrested by the addition of 50 mM L tryptophan. The residual activity was determined under standard assay conditions. NBS mediated inactivation was monitored by measuring the decrease in absorbance at 280 nm. Tryptophan was determined by titration with NBS (Spande and Witkop 1967).The number of tryptophan residues

oxidized (n) per mole enzyme was calculated from the relationship:

$$n = (1.31 \times \Delta_{280}) / (5500 \times \text{molarity of enzyme})$$

where Δ_{280} is the decrease in absorbance at 280nm, $5500\text{M}^{-1}\text{cm}^{-1}$ is the molar extinction co-efficient of tryptophan at pH 4.5 and 280 nm and 1.31 is an empirical factor based on oxidation of model tryptophan peptides (Patchornik et al.1958).

$M_r=67$ kDa was used for calculating molarity of the enzyme β Fructofuranosidase from *K. marxianus* NCYC 2675.

Protection of the enzyme against inactivation by NBS (20 μM) and HNBBBr (40 mM) was determined by incubating the enzyme with varying concentrations of substrate sucrose or products glucose or fructose prior to treatment with modifying reagents under the reaction conditions.

Circular dichroism analysis

Both the NBS and HNBBBr modified β Fructofuranosidase samples were dialyzed to remove excess reagent and then the CD spectra were recorded. Untreated enzyme is used to record the native spectra.

RESULTS AND DISCUSSION

Purification and characterization

The homogenous enzyme preparation with specific activity of 2166 units/mg and 65% yield was used in further studies.

The purified enzyme β Fructofuranosidase from *K. marxianus* NCYC 2675 moved as a single band on 7.5% native PAGE (**Fig 1a**) and SDS-PAGE (**Fig 1b**). The SDS-PAGE run of the purified enzyme showed a molecular weight of 67kDa. Sugar was determined from the standard graph and the percentage of sugar in the protein was calculated. No significant level of sugar was obtained in the protein. The standard graph was prepared using mannose since a majority of the glycosylated invertases have a high content of mannose oligosaccharides (Gascon and Lampen 1968). The enzyme from *K. marxianus* NCYC 2675 was found to be a non glycosylated enzyme.

Isoelectric focusing showed a single band at pH 3.7.

Chemical Modification of Tryptophan using NBS and HNBBBr

Both NBS and HNBBBr inactivated the enzyme β Fructofuranosidase rapidly at pH 4.5 as shown in **Fig 3.a** and **3.b**. The enzyme activity in the presence of 60 μM NBS was 25% at the end of 40 minutes test period, while the residual activity in the presence of 40mM HNBBBr was 40% at the end of 30 minutes of incubation.

The plots of the logarithm of residual activity versus time of incubation with the NBS and HNBBBr were linear throughout the test period of 40 minutes and 30 minutes respectively at all the concentrations of the modifier that were used, indicating pseudo first-order kinetics of inactivation by both reagents. In absence of modifiers, no loss in enzyme activity was observed. The individual slopes of the plots were calculated to determine the respective first-order rate constant K_{app} . The reaction order (n) with respect to the tryptophan modifying reagents determined from the plots of $\log[K_{app}]$ versus \log reagent concentration $[M]$ gave value of $n=1.06$ for NBS and $n=1.04$ for HNBBBr.

These values indicate that the modification of a single tryptophan residue results in the inactivation of a mole of the enzyme (inset of **Fig 3a** and **Fig 3b**). The protective action of substrate sucrose and products of invertase namely glucose and fructose on the inactivation of the enzyme by NBS and HNBBBr is shown in **Table 1**. In the presence of sucrose (50 mM) residual activity was 75% of the initial activity after 30 min incubation with NBS (60 μM) and 81% after incubation with HNBBBr (40 μM), compared with 25% and 30% respectively in the absence of substrate.

Glucose (50mM) gave corresponding values of 63% and 80% activity for NBS and HNBBBr respectively. Fructose protected the enzyme almost 60% against inactivation of the enzyme by NBS (60 μM) in 30 min. Under the conditions of the experiment fructose protected the enzyme 83% against inactivation by HNBBBr (40 mM).

The kinetic evidence for the involvement of a single tryptophan residue and the protective action of the substrate and products against inactivation of β Fructofuranosidase from *K. marxianus* NCYC 2675 by tryptophan modifying reagents are indicative of

the presence of the tryptophan moiety at or near the active site of the enzyme.

The tryptophan residues in *K. marxianus* NCYC 2675 β Fructofuranosidase were oxidized with stepwise addition of NBS. The number of tryptophan residues oxidized was determined from $A_{280\text{nm}}$ and the molar ratio calculated using M_r value of 67,000 for the enzyme as described in **Materials and methods**. **Fig 4** shows the effect of NBS on enzyme activity after each addition of NBS. There was a progressive decrease in absorption at 280 nm as well as increase in extent of inactivation. By extrapolating

the initial linear portion of the plot to zero activity, the number of tryptophan moieties oxidized per mole of enzyme was found to be 1.0. This usually gives the number of residues modified when the enzyme is completely inactivated.

Circular Dichroism analysis:

The shape of the CD spectra (**Fig 5**) of modified β fructofuranosidase was similar to that of untreated enzyme indicating that no serious structural changes occurred on treatment with NBS and HNBBBr.



Figure 1a : Native PAGE

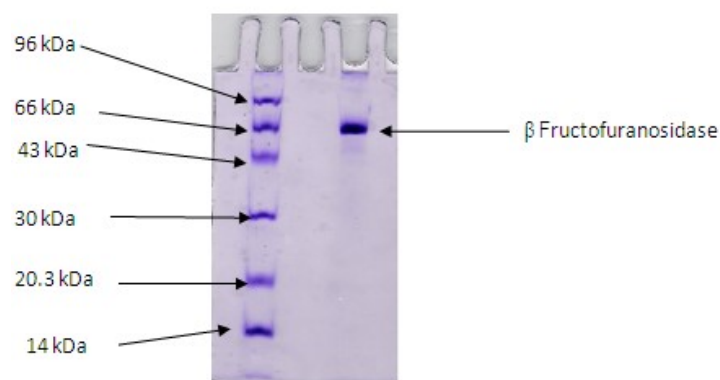


Figure 1b: SDS-PAGE

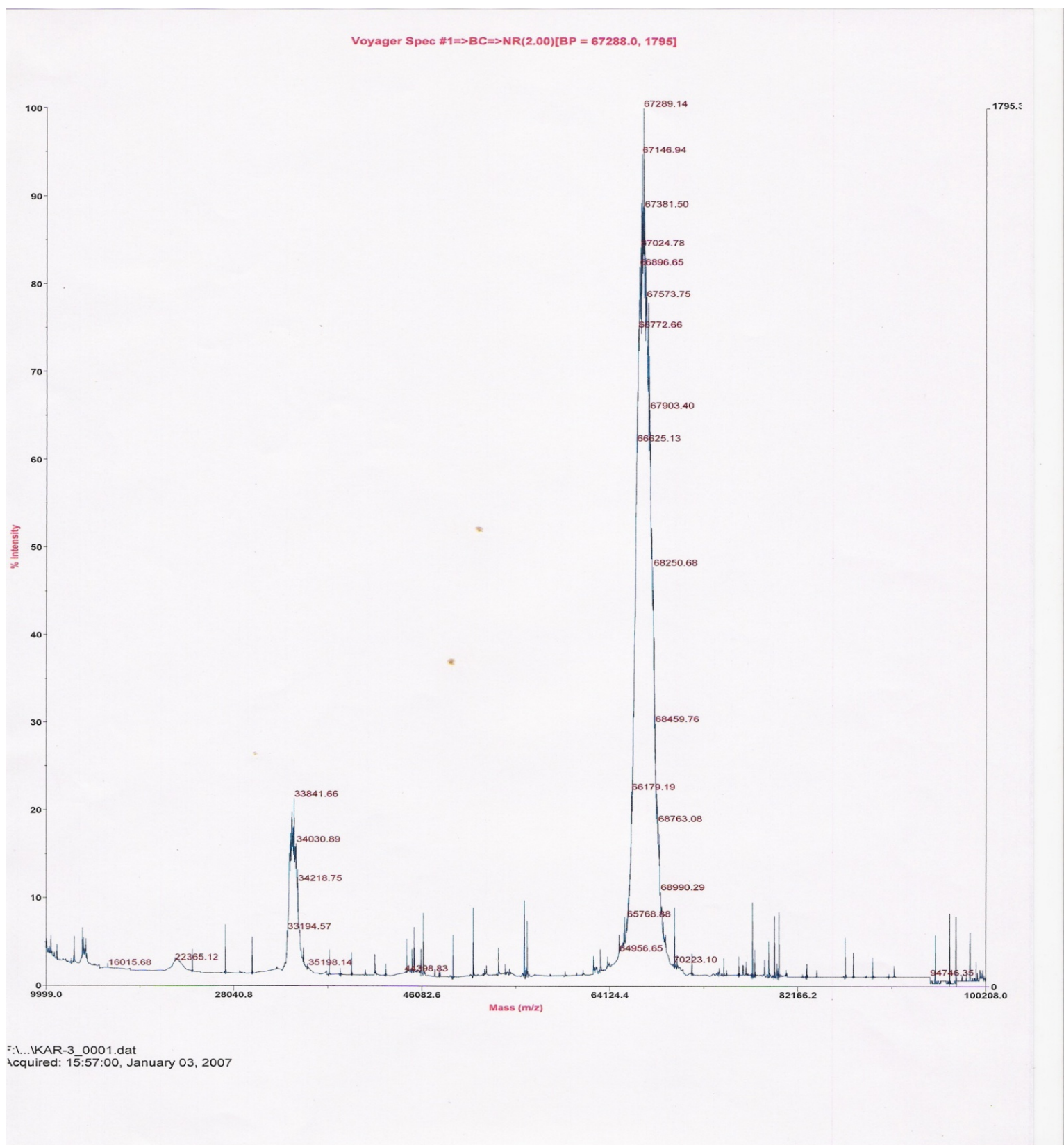


Figure 2: MALDI TOF
MALDI analysis of the purified protein from K. marxianus NCYC 2675 showing molecular mass of 67 kDa

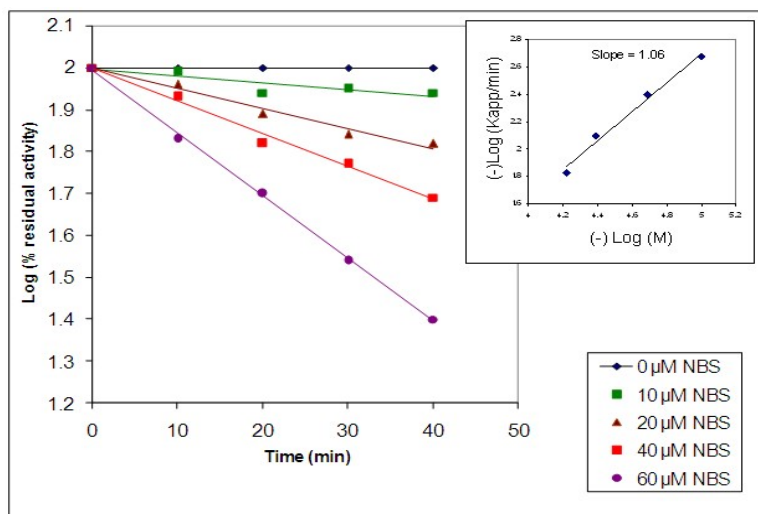


Figure 3a: Inactivation of β Fructofuranosidase from *K. marxianus* NCYC 2675 by NBS

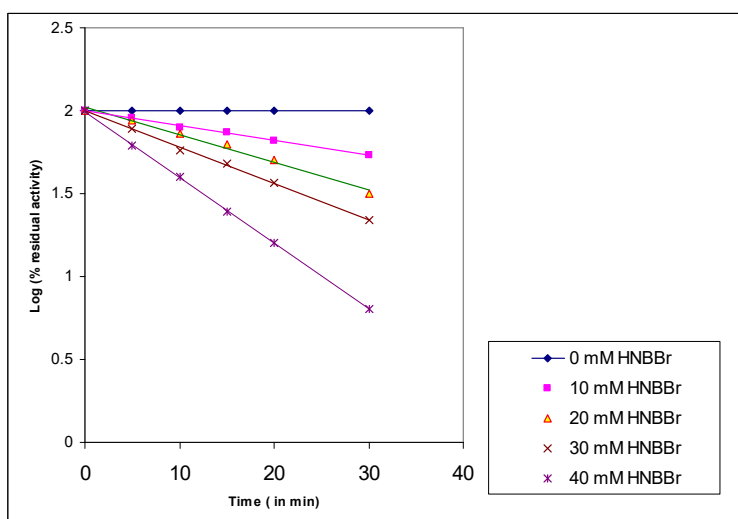


Figure 3b: Inactivation of β Fructofuranosidase from *K. marxianus* NCYC 2675 by HNBBR

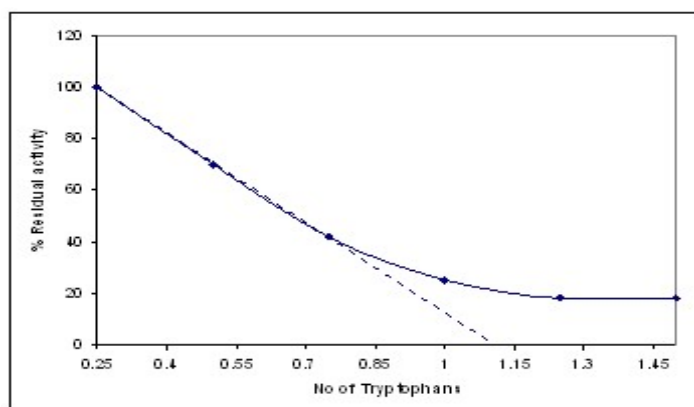


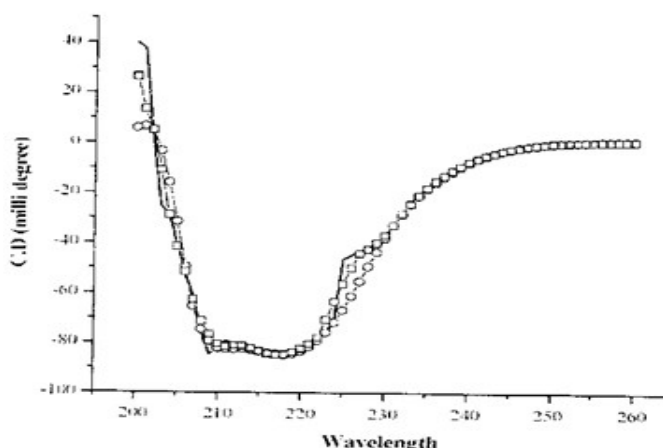
Figure 4: Titration with NBS of accessible tryptophan residues in *K. marxianus* β Fructofuranosidase

Figure 5: The CD spectra of untreated invertase(-) and that chemically modified with NBS(○) and HNBBBr(□). The spectra were recorded on a JASCO-710 spectropolarimeter from 190 to 260 nm using 1cm path length at 25 C and an enzyme concentration of 0.5mg/ml in 50mM buffer, pH 4.5. X axis: wavelength, Y axis: ellipticity.

Table 1: Protection of β Fructofuranosidase against inactivation by tryptophan-specific reagent

Treatment	Enzyme activity (% of the initial activity)
None	100
N-Bromosuccinamide (60 μ M)	25 \pm 1
Sucrose(60mM)+N-bromosuccinimide (60 μ M)	75 \pm 2
Glucose (60mM) +N-bromosuccinamide (60 μ M)	63 \pm 3
Fructose (60mM) +NBS	60 \pm 2
HNBBBr (40mM)	30 \pm 1
Sucrose (60mM) +HNBBBr (40mM)	81 \pm 2
Glucose (60mM) +HNBBBr (40mM)	80 \pm 4
Fructose (60mM) +HNBBBr (40mM)	83 \pm 3

CONCLUSION

In the present work, an intracellular invertase from the yeast *K. marxianus* NCYC 2675 was purified to homogeneity by ammonium sulphate fractionation and two successive octyl sepharose affinity chromatographies. The final step of purification yielded an enzyme with specific activity of 2116 μ moles /min/mg. The activity yield was 65%. The homogeneity of the purified enzyme had been established by PAGE, SDS-PAGE and IEF. Single protein bands were obtained in all the procedures.

The molecular weight was estimated to be 67 kDa as determined by gel chromatography, SDS-PAGE and MALDI-TOF. Glycoprotein estimation indicated non-glycoprotein nature of the enzyme. The mechanism of action and the nature of the essential amino acid residues at or near the active site of *K. marxianus* NCYC 2675 have been reported for the first time. The present studies indicate the involvement of a single tryptophan residue in the active site. The evidences are the kinetics of inactivation by NBS and HNBBBr and protection from NBS inactivation by substrate.

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