

## Increased Yield of Enzymatic Synthesis by Chromatographic Selection of Different N-Glycoforms of Yeast Invertase

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Received: 20/04/2020; Revised: 20/09/2020; Accepted: 28/09/2020

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/elps.202000092](#).

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**Keywords:** Enzyme stability / Glycoform separation / Monolithic supports / N-glycosylation / Organic solvent

**Abbreviations:** APT, attached proton test; COSY, correlation spectroscopy; EINV, invertase glycoforms; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple-quantum correlation; MF, methyl  $\beta$ -D-fructofuranoside

### Abstract

Invertases are glycosidases applied for synthesis of alkyl glycosides that are important and effective surfactants. Stability of invertases in the environment with increased content of organic solvent is crucial for increase of productivity of glycosidases. Their stability is significantly influenced by N-glycosylation. However, yeast N-glycosylation pathways may synthesize plethora of N-glycan structures. A total natural crude mixture of invertase glycoforms (EINV) extracted from *Saccharomyces cerevisiae* was subfractionated by anion-exchange chromatography on industrial monolithic supports to obtain different glycoforms (EINV1–EINV3). Separated glycoforms exhibited different stabilities in water-alcohol solutions that are in direct correlation with the amount of phosphate bound to N-glycans. Observed differences in stability of different invertase glycoforms were used to improve productivity of methyl  $\beta$ -D-fructofuranoside (MF) synthesis. The efficiency and yield of MF synthesis was improved more than 50% when the most stable glycoform bearing the lowest amount of phosphorylated N-glycans is selected and utilized. These data underline the importance of analysis of glycan structures attached to glycoproteins, demonstrates different impact of N-glycans on the surface charge and enzyme stability in regard to particular reaction environment, and provide a platform for improvement of yield of industrial enzymatic synthesis by chromatographic selection of glycoforms on monolithic supports.

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## **1 Introduction**

Industrial enzymes and therapeutic proteins are widely produced in yeast cells [1]. Baker's yeast *Saccharomyces cerevisiae* production system is commonly used because it is non-pathogenic and non-toxicogenic, robust, rapid and it can be scaled-up on a simple way. Extracellular proteins, expressed in natural yeast strains can be highly N-glycosylated and they can also exist in many different glycoforms [2]. Different N-glycan structures attached to different positions in AA sequence may affect protein stability, enzymatic activity as well as biological properties, thus need to be examined [3–10]. However, elucidation of structure-function relation of glycoproteins is very challenging task since production of proteins bearing particular glycan is still hardly feasible [11–12]. Moreover, precise structure of certain high molecular weight glycan, such as high mannose (*vide infra*), remains unresolved due to poor resolution of separation of glycan structures and/or selectivity/sensitivity of analytical procedures [13]. Chromatographic subfractionation of glycoforms is one of the major methods for production/isolation as well as for narrowing glycoform populations and/or for control of glycosylation heterogeneity [5, 14–16]. Understanding the effect of different glycans and their position in AA sequence on surface charge and hydrophobicity is essential for biochemical activity and design of separation/purification processes [5, 7, 17]. Application of hydrolytic enzymes in water-organic solutions for chemical synthesis has numerous industrially attractive advantages over classical organic synthesis: operate at room temperature, as well as under mild, non-toxic and environmentally safe conditions, no need for functional group protection in order to achieve regioselectivity, and products are

stereochemically defined [18, 19]. Utilization of most enzymes for synthesis in water-organic solutions or in pure organic solvents is restricted due to their decreased activity and stability under those conditions. Detailed knowledge of the structure of enzyme and effect of glycosylation on structure stability will enable a better understanding of the mechanism of enzyme stabilisation and/or inactivation. This is necessary for better control over the deactivation process and engineering of enzymes with increased stability [20, 21].

Yeast *S. cerevisiae* invertase ( $\beta$ -fructofuranoside fructohydrolase, EC 3.2.1.26) is an important enzyme in biotechnology, as well as in both food and pharmaceutical industries. It catalyzes hydrolysis of sucrose to equimolar mixture of glucose and fructose. Furthermore, this enzyme can be used for synthesis of fructo-oligosaccharides [22], and it can be also applied for synthesis of alkyl  $\beta$ -fructosides using water-organic alcohol mixtures (methanol and ethanol) [23]. The *S. cerevisiae* cells produce two forms of invertase – the intracellular non-glycosylated and the extracellular (“external”) heavily N-glycosylated form. External invertase is secreted into the periplasmic space that is situated between the cell membrane and the cell wall. Both forms share the same AA sequence (MW 58.6 kDa) that contains 14 potential N-glycosylation sites. In external invertase, N-glycosylation is found on 13 sites [24, 25]. The apparent molecular weight of the external invertase homodimer varies due to differences in glycosylation and the average value is between 200–270 kDa. All N-glycans are of high-mannose type with a length that varies from 3 to more than 70 mannose units [13, 24–27]. Certain high mannose N-glycans can contain phosphate groups bound to mannose in diester form, see Figs. 1a,c,d [13, 24, 28]. Additionally, small amount of phosphate groups bound to mannose in monoester form was observed, see Fig. 1b, wherein phosphate in diester form with more than 95% is predominant [28]. Phosphate groups are also one of the sources of polymorphism of this enzyme and after separation by the isoelectric focusing at least seven bands of different pI values were observed [29, 30]. We have developed chromatographic

procedure for purification of external invertase isoforms [14]. The purified isoforms contain different amounts of phosphate groups covalently attached to the N-glycans [28]. These forms exhibit the same catalytic properties (e.g.,  $K_m$  for sucrose) and the pH optimum [14]. At the other hand, their pI values, thermal stability and chemical reactivity towards epoxy-activated Eupergit C beads for enzyme immobilization are different [14], as well as structural features and thermodynamic stability in guanidinium-chloride solutions [31]. These differences correlate with surface negative charge density and the content of phosphate groups bound to high-mannose N-glycans [28].

Alkyl glycosides are effective non-ionic surfactants important for their use as detergents, foodstuffs, as well as products for the cosmetic and pharmaceutical industry [32, 33]. They exhibit anti-microbial activity [34] and are also considered to be safe for the environment due to their rapid biodegradation [32, 33]. Anomerically pure glycosides can be synthesized in one step using glycosidases (EC 3.2.1.) via reversed hydrolysis (thermodynamic control) or transglycosylation (kinetic control) [35, 36]. In such a way, methyl  $\beta$ -D-fructofuranoside (MF) can be synthesized using invertase in a kinetically controlled transglycosylation process [23, 37].

The application of invertase for the synthesis of alkyl  $\beta$ -fructosides is restricted by the enzyme's stability in water-alcohol mixtures [38]. Concentration of methanol higher than 20% (v/v) induce irreversible deactivation of external invertase while concentrations higher than 50% (v/v) lead to aggregation and precipitation of the enzyme [14, 38]. Increasing the concentration of methanol favours the alcoholysis according to reaction 2, shown in Fig. 2a, and it also disfavours hydrolysis of MF [23]. In order to achieve higher yields, the water activity has to be as low as possible. The reason is that under such conditions the competing reaction of hydrolysis will be minimized. Such favourable environment can be achieved by increasing methanol concentration. Therefore, it is necessary to have an invertase capable of

maintaining a high degree of activity in the highest possible concentration of this organic solvent.

In this paper, we designed chromatographic subfractionation, suitable for industrial scale, of crude external invertase N-glycoform mixture to obtain different populations of N-glycoforms. Stability of separated invertase glycoform populations carrying various glycans under different methanol concentrations was also determined, and their glycan components were compared. The efficacy of methyl  $\beta$ -D-fructoside synthesis by different invertase glycoform populations was studied. Since many enzymes of importance for biotechnology are glycosylated, and biologically synthesized as mixtures of glycoforms, we demonstrate influence of N-glycosylation of surface charge and importance of chromatography in adequate selection of enzyme glycoform populations in order to improve the yield of enzyme catalyzed reactions.

## 2 Materials and methods

External invertase was purified from *S. cerevisiae* (baker's yeast Alltech Fermin Senta, Serbia) according to the procedure described previously [14]. Commercial external invertase (Grade VII from baker's yeast) was from Sigma-Aldrich (St. Louis, MO, USA). Methanol was LC grade from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

### 2.1 Isolation of external invertase glycoforms

The lyophilisate of external invertase was dissolved in 50 mM sodium acetate buffer, pH 5.5, and loaded onto an anion-exchange CIM-QA disc (bed volume 0.34 mL) (BIA Separations, Ajdovščina, Slovenia) equilibrated in the same buffer, using fast protein liquid chromatography system (Knauer, Berlin, Germany). Four glycoforms (EINV1–EINV4) were separated by applying a step gradient elution as described previously (Andjelkovic et al. 2010) with one change in this procedure: the first elution step was performed at buffer

conductivity of 5.5 mS/cm (instead of 4.9 mS/cm). Each peak was re-chromatographed, on the same column. In a separate run, the sample of the total external invertase (EINV) was obtained by pooling all fractions containing invertase activity, and subsequently desalted and concentrated by ultrafiltration (ultrafiltration membrane with a cut off of 10 kDa, Millipore, Burlington, MA, USA). The samples were stored at  $-20^{\circ}\text{C}$  until used.

## 2.2 Invertase activity assay and concentration determination

Invertase (25  $\mu\text{L}$ ) was added to 0.3 M sucrose solution in 50 mM sodium acetate buffer (475  $\mu\text{L}$ ), pH 5.0. After 5 min at  $25^{\circ}\text{C}$  the reaction was terminated by addition of 2,4-dinitrosalicylic acid reagent (500  $\mu\text{L}$ ) and the mixture was boiled in a water bath for 5 min. Before measuring absorbance at 540 nm, 4 mL of deionized water was added. A standard curve was obtained with different concentrations (0.5–10 mM) of an equimolar mixture of D-glucose/D-fructose in 50 mM acetate buffer, pH 5.0. One unit of invertase activity (U) corresponds to the amount of enzyme that catalyses the hydrolysis of 1  $\mu\text{mol}$  of sucrose per 1 min under described assay conditions. Concentration of pure invertase was obtained by measuring absorbance at 280 nm ( $A_{280\text{ nm}}=2.25$  for 1 mg/mL invertase solution).

## 2.3 Fluorescence spectroscopy

Fluorescence emission spectra were recorded using a spectrofluorimeter FluoroMax 4 (Horiba Jobin Ivon, Kyoto, Japan), equipped with a cuvette of 1cm path length. The effect of methanol on external invertase glycoforms was assessed by measuring the intrinsic emission fluorescence from 310 to 390 nm using excitation at 280 nm. Slit widths were set at 5 nm both for excitation and emission. The concentration of enzyme was 0.016 mg/mL in 50 mM acetate buffer pH 5.0.

## 2.4 Analysis of N-glycans

### 2.4.1 Glycan release and labelling

The analytical procedure was performed as previously reported [39]. Briefly, the samples were dried and denatured with the addition of SDS (sodium dodecyl sulfate, Invitrogen, Carlsbad, CA, USA) and by incubation at 65°C. The excess of SDS was neutralized with Igepal-CA630 (Sigma-Aldrich) and N-glycans were released following the addition of PNGase F (Promega, Fitchburg, WI, USA) in PBS. The released N-glycans were labelled with 2-aminobenzamide (2-AB). Free label and reducing agent were removed from the samples using HILIC-SPE. Glycans were eluted with ultrapure water and stored at -20 °C until use.

### 2.4.2 Ultra-performance liquid chromatography

Fluorescently labelled N-glycans were separated by HILIC on an Acquity UPLC instrument (Waters, Milford, MA, USA) consisting of a quaternary solvent manager, sample manager, and an FLR fluorescence detector set with excitation and emission wavelengths of 250 and 428 nm, respectively. The instrument was controlled by Empower 3 software, build 3471 (Waters). Labelled N-glycans were separated on a Waters BEH Glycan chromatography column, 100 × 2.1 mm i.d., 1.7 µm BEH particles, with 100 mM ammonium formate, pH 4.4, as solvent A and acetonitrile as solvent B. For separation a linear gradient of 30–100% solvent A at flow rate of 0.561 ml/min in a 52 min analytical run was used. Data processing was performed using an automatic processing method with a traditional integration algorithm, after which each chromatogram was manually corrected to maintain the same intervals of integration for all the samples. The chromatograms were all separated in the same manner into 32 peaks [3].



Separations of oligosaccharide chains that were strongly bound to the column were attempted on other HILIC columns, namely ZORBAX RRHD 300-HILIC (Agilent, Santa Clara, CA, USA) and GlycoSep C HPLC column (Prozyme, Hayward, CA, USA).

#### 2.4.3 MALDI TOF/TOF MS

The molecular masses of N-glycans and their isobaric monosaccharide compositions were determined by MALDI TOF/TOF MS. Briefly, UPLC fractions corresponding to glycan peaks of interest were collected, dried and resuspended in 2–5  $\mu\text{L}$  of ultra-pure water depending on the length of the fraction. Samples were cleaned up using cotton HILIC - solid phase extraction and the N-glycans were dried, resuspended in 2  $\mu\text{L}$  of ultra-pure water and spotted onto a MTP AnchorChip 384 BC MALDI target (Bruker Daltronics, Bremen, Germany), mixed on plate with 1  $\mu\text{L}$  of matrix solution (5 mg/mL 2,5-dihydroxybenzoic acid, 1 mM NaOH in 50% ACN) and left to air dry. Recrystallization was performed by adding 0.2  $\mu\text{L}$  of ethanol to each spot. Analyses were performed in reflector positive mode on an UltraFlexreme MALDI-TOF/TOF MS equipped with a Smartbeam-II laser and Flexcontrol 3.4 software Build 119 (Bruker Daltonics). The instrument was calibrated using a human plasma N-glycome standard. A 25 kV acceleration voltage was applied after a 140 ns extraction delay. A mass window of  $m/z$  1000 to 5000 with suppression up to  $m/z$  900 was used for N-glycan samples. For each spectrum, 10,000 laser shots were accumulated at a laser frequency of 2000 Hz, using a complete sample random walk with 200 shots per raster spot. Tandem mass spectrometry was performed on the most abundant peaks via laser-induced disassociation with post acceleration by LIFT.

#### 2.5 Enzyme stability in methanol-water mixtures

Samples of the external invertase isoforms (15 U/ mL) in deionized water were incubated at 40°C in the presence of varying concentrations (v/v) of methanol. Aliquots of 25  $\mu\text{L}$  were

taken every 15 min and the residual activity measured using the described conditions of invertase assay (*vide supra*).

## 2.6 Enzymatic synthesis of methyl $\beta$ -D-fructoside

Synthesis was performed in 50 mM acetate buffer, pH 5, at 40°C in a thermo block with constant shaking. The initial concentration of reactants was 0.3 M sucrose and 30% (v/v) methanol in total reaction volume of 10 ml. Before addition of the enzyme, the reaction mixture was incubated for 5 min at 40°C. Invertase was added to achieve a concentration of enzyme activity of 15 U/ml. Aliquots of 1 ml were taken every 15 min. Each aliquot was immediately incubated at 65°C for 1 min to inactivate the enzyme. After inactivation, the enzyme was removed by ultrafiltration with 3 kDa cut-off (Millipore). Complete filtrate was collected, dried under vacuum (SpeedVac, Eppendorf, Hamburg, Germany) and used for NMR quantification.

## 2.7 NMR measurements

The one- and two dimensional homo- and heteronuclear  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded with a Bruker AV-600 spectrometer (Bremen, Germany), operating at 600.133 MHz for the  $^1\text{H}$  nucleus and 150.917 MHz for the  $^{13}\text{C}$  nucleus. Measurements were performed in DMSO- $d_6$  solutions with  $\text{SiMe}_4$  as an internal standard. The measurement techniques used were:  $^1\text{H}$ , attached proton test (APT), homonuclear correlation spectroscopy (COSY), heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC). All the experiments were performed in the following conditions: solvent (DMSO- $d_6$ ), concentration (0.07 g in 0.6 ml of solvent), temperature (298 K), number of scans 256 for  $^1\text{H}$ , 1200 for  $^{13}\text{C}$ -APT, 4 for  $^1\text{H}$ - $^1\text{H}$  COSY, 16 for  $^1\text{H}$ - $^{13}\text{C}$  HMQC, 16 for  $^1\text{H}$ - $^{13}\text{C}$  HMBC. As an external standard Anisole (methoxybenzene) was used for determining the ratio of the methylated fructose in samples. Anisole was sealed in a glass capillary ( $\varnothing$  1 mm), immersed into a standard 5 mm NMR tube with dissolved samples.

### 3 Results

#### 3.1 Separation of invertase glycoforms

Four glycoform populations (EINV1–EINV4) of external invertase were isolated either from the commercial preparation of the enzyme or extracted from local baker's yeast (*S. cerevisiae*). All experiments were performed with both samples and they were identical in the range of experimental error. Results presented in this publication are those obtained with external invertase purified from local baker's yeast *S. cerevisiae*. Our previous separation protocol [14] was optimized, and the isolation of four invertase glycoform populations was now achieved by use of the preparative anion-exchange QA CIM monolithic column. Step elution gradient was thoroughly optimized in regard to the salt level (conductivity) of elution buffer for each elution step. High purity of separated glycoforms was confirmed by standard tris/glycine SDS-PAGE, see Fig. 2c. Specific activities of purified preparations of external invertase glycoforms are listed in supplementary Table S1.

The glycoform EINV2 is the most abundant glycoform purified from the sample of total external invertase, see chromatogram in Fig. 2b. The abundance of glycoform population EINV4 (peak with invertase enzyme activity following peak 3, chromatogram at Fig. 2b) was less than 5%. The difference between glycoforms can be observed as different electrophoretic mobility in native-PAGE, see Fig. 2d.

#### 3.2 Fluorescence spectroscopy analysis of methanol effect on invertase glycoforms

Fluorescence of invertase predominately comes from tryptophan. According to AA sequence (UniProt P00724) invertase contains 16 tryptophan residues. The intensity of fluorescence is influenced by local environment around the tryptophan residues. Conformational changes of separated glycoforms, which were used in further experiments, (EINV1–EINV3) are influenced by different methanol concentration. That was demonstrated by fluorescence spectroscopy.

The fluorescence spectra of external invertase glycoforms in 50 mM sodium acetate buffer, pH 5.0, are identical (results not shown). The fluorescence intensity, at 340 nm, of the 3 glycoform populations and the total external invertase (EINV) during incubation in 20% (v/v) methanol (see Fig. 3a), slightly decreases (less than 10%) over a course of 90 min. As shown in Fig. 3a, different stability of separated fractions was observed when samples were incubated in 30% (v/v) methanol. After 90 min of incubation fluorescence intensity for glycoform EINV1 decreased by about 10%, while for both other forms EINV2 and EINV3 the intensity decreased by 40%. The fluorescence intensity of the total invertase, sample before subfractionation, (EINV) decreased by about 30%. A methanol concentration of 40% (v/v) induces a rapid decrease of fluorescence intensity of all samples, see Fig. 3a.

The wavelength shifts of emission maxima of tryptophan fluorescence during incubation in methanol are presented in Fig. 3b. The emission maximum of the various glycoforms and EINV, after 90 min incubation in 20% (v/v) methanol at 40°C is slightly red shifted, see Fig. 3b. A clear distinction between EINV1 and other glycoforms and EINV is observed after incubation in 30% (v/v) methanol at 40°C (Fig. 3b). After 90 min of incubation the emission maximum of EINV1 is slightly red shifted while the red shift is three times higher for the fractions EINV2, EINV3 and EINV. In 40% (v/v) methanol the maximum emission wavelength shift of all invertase samples is very fast and significantly red shifted to the value that remains constant after 90 min, see Fig. 3b. At the same time, the intensity decreases gradually, see Fig. 3a.

### 3.3 Effect of methanol on enzymatic activity of invertase glycoforms

The effect of different concentrations of methanol on the activity of external invertase glycoforms and the natural mixture of the enzyme forms (EINV) was additionally analyzed. The activity in 20% methanol after 90 min incubation at 40°C was decreased by 15% for the fraction EINV1 and 20% for other three other samples, namely EINV2, EINV3 and EINV

(figure not shown). In 30% methanol the glycoforms, and the mixture of the enzyme forms before fractionation (EINV) significantly differ in their stability (see Fig. 3a) and activity (see Fig. 4). The obtained results showed that the fraction EINV1 is distinctly more stable than other three fractions, EINV2, EINV3 and EINV. The EINV1 activity at the end of incubation period represented about 65% of the starting activity before incubation of this isoform. On the other hand, the activity of other glycoforms rapidly decreased and after 90 min incubation there was only a weakly detectable invertase activity remaining. In 40% methanol at 40°C all samples lose their native structure, see Fig. 3, and activity (data not shown) in few minutes.

### 3.4 N-glycan analysis of external invertase

Oligosaccharide chains were enzymatically released by PNGaseF that selectively cleaves N-glycans carrying no alpha 1,3 linked fucose. Deglycosylated protein were analysed by native-PAGE, as shown in Fig. 2d. Identical electrophoretic mobility of deglycosylated proteins was observed.

Released glycans were labelled with 2-AB and separated on a HILIC column using a linear-gradient elution, (see Materials and methods and Fig. 5). Glycan fractions that were separated during the first 16 min were collected and their structure was analysed by MALDI-TOF/TOF MS. Mass spectrometry analysis confirmed that these fractions contain core high-mannose N-glycans with 3 to 9 monosaccharide units attached to GlcNAc<sub>2</sub> (see Fig. 5 inset). These peaks can be found in all three analysed preparations of glycoforms after PNGaseF treatment. On the other hand, high mannose glycans that contain 10 to >70 mannose units, are strongly bound to the column. They eluted after 30 min, see Fig. 5 and could not be separated.

Complete separation of each individual glycan molecule is necessary for elucidation of their structure. Separation of these high molecular weight glycans cannot be achieved with other HILIC columns (ZORBAX and GlycoSep) that were also used for parallel analysis (data not shown). Nevertheless, the chromatographic profiles of the dominant mannose chains

(composed of 10 to >70 mannose units) released from external invertase glycoforms are completely different in term of peak positions (Fig. 5, peaks after 30 min).

### 3.5 Enzymatic synthesis of methyl $\beta$ -D-fructoside

The reaction conditions used in this study were those found by previous studies as optimal concerning yield of MF [23, 38]. Studies of enzymatic synthesis of MF were performed with different glycoforms of external invertase. Enzymatic synthesis was performed under high methanol concentration (30% v/v) in order to increase yield and efficacy. The structure of the product (MF) was confirmed by NMR measurements, see supplementary Figs. S1 and S2.

Conversion of sucrose to MF was determined from the intensity ratio of protons at 3.19 and 4.25 in  $^1\text{H}$  NMR, corresponding to MF and fructose, respectively. Enzymatic reaction in 30% v/v methanol enables  $44 \pm 5\%$  of degraded sucrose being converted to MF. All tested glycoforms exhibited similar conversion ratio until maximal concentration of MF was reached. When maximal concentration of MF is reached further ratio of conversion of sucrose to MF decrease due to hydrolysis of MF (see Fig. 2. reaction [2]), as well as due to inactivation of enzyme (see Figs. 3 and 4).

Quantification of MF was also performed by NMR measurements. The ratio of anisole proton signal intensity against methoxy proton signal intensity of products is quantified from  $^1\text{H}$  NMR peak picking as absolute proton signal intensity. Time courses of formation of MF are presented in Fig. 6a. Initially the concentration of MF increases, but after 45 min it starts to decrease. As shown in Fig. 6b, that highest amount of MF can be obtained when enzyme glycoform EINV1 is used. But, the concentration of MF after 90 min of enzymatic reaction is lowest when enzyme glycoform EINV1 is used, see Fig. 6a.

The highest yield was achieved with glycoform EINV1 after 45 min, see Fig. 6, under the given reaction conditions.

#### 4 Discussion

Baker's yeast external invertase is highly glycosylated, and the preparation of this enzyme is a mixture of different glycoforms. This mixture can be separated into glycoform populations by anion-exchange chromatography that is also suitable for industrial application. Optimal subfractionation was achieved using a step elution gradient on QA-CIM discs. Low abundance of glycoform population EINV4 (less than 5%) did not nominate it for further consideration in this research.

The difference in surface negative charge density between glycoforms enables their separation by strong anion exchange chromatography. Different electrophoretic mobility in native-PAGE, see Fig. 2d, confirms that glycoforms have different surface negative charge density.

Slight decrease of fluorescent intensity presented at Fig. 3a indicates that 20% methanol has only a small impact on tryptophan environment. In 30% methanol fluorescent intensity decrease is substantially different between isoforms, Fig. 3a. Decrease of 10% in the case of EINV1 indicate that under these conditions the structure of EINV1 is still well preserved unlike structure of both other forms used in this experiment, namely EINV2 and EINV3 where fluorescence decrease of 40% was observed. Since EINV is a mixture of all invertase glycoforms, decrease of fluorescence of 30% is consistent with finding that EINV1 is not the most abundant glycoform in the natural mixture. The presented results for EINV are in agreement with previous findings for a sample of the total external invertase [38].

Alongside with changes of fluorescence intensity, observed shift of the maximum emission wavelength (Fig. 3b) may indicate that two different processes could be present in methanol. This is consistent with previous findings that 40% methanol as a solvent, beside deactivation, additionally leads to aggregation of the enzyme [38]. Fast red shift of the maximum emission wavelength that is accompanied with gradual change of fluorescence intensity, may also

indicate that structural changes that affect tryptophan residues are not the same as those that affect enzyme activity.

Based on enzyme activity and fluorescence measurement (see Fig. 3a) during 90 min it can be assumed that the tertiary structure of external invertase in 20% methanol is mostly preserved during this time. The obtained enzyme activity results in the presence of 30% methanol showed that the fraction EINV1 is distinctly more stable than other three fractions, EINV2, EINV3 and EINV, keeping its activity over 90 min of incubation at 40°C. Finally, 40% methanol induces fast and fatal effect on activity of all invertase glycoforms. The highest tolerated methanol concentration concerning invertase stability is 30% (v/v) under given conditions. Under these conditions, glycoform EINV1, that contains lowest amount of negative charged phosphate groups, exhibits significantly higher stability.

Identical electrophoretic mobility of deglycosylated proteins confirms previous findings that external invertase contains exclusively N- bound glycans [14, 25]. Moreover, this indicates that the observed differences between purified molecular forms of external invertase are a consequence of differential glycosylation. Hence, it can be stated that different glycoforms of external invertase were fractionated. Nevertheless, the presence of microheterogeneity of glycan structures, which also can be named as glycoforms, in each preparation of individual glycoforms cannot be excluded.

Analysis of glycans was performed in order to confirm that the separated molecular forms of external invertase are differently glycosylated. As was observed at chromatogram (Fig. 5) glycan fractions eluting after 30 min represent dominant N- glycan chains. These dominant high mannose glycans consist of 10 to >70 mannose units and they also contain phosphate bound as di- and mono- esters, see Fig. 1c,d [13, 24, 26, 28]. A difference in chromatographic profiles, under the same chromatographic and sample preparation conditions, suggests that



these dominant high mannose N- glycans have different structures. Their separation and structural determination is a challenging task that is a subject of our future research.

External invertase glycoforms exhibit different negative surface charge density which increases in the order  $EINV1 < EINV2 < EINV3$ , based on elution chromatogram and mobility in native PAGE, see Fig. 2b,d. The glycoform EINV1 exhibits the lowest negative surface charge density of all glycoforms. The stability of this glycoform in 30% v/v methanol (the dielectric constant is decreased in comparison to water) is highest comparing to the other glycoforms of this enzyme.

The different surface charge density of invertase glycoforms depends on the amount and distribution of phosphate groups covalently attached to the polymannan components as it was described in our previous work [28]. This distribution is important for thermodynamic stabilization of the glycoprotein [40, 41]. Both, different distribution and amount of the phosphorylated polymannan component could also be responsible for the observed differences in stability between glycoforms. However, further research is necessary in order to confirm this hypothesis.

The effect of charged glycans on stability of invertase glycoforms was used to improve efficacy of methyl  $\beta$ -D-fructoside synthesis. Enzymatic synthesis of MF, and other alkyl fructosides, according to reaction [2] Fig. 2a, can be performed in two ways, using as reactants either sucrose and methanol or fructose and methanol. The reaction between sucrose and methanol is irreversible under reaction conditions used for enzymatic synthesis. Yield of MF in this reaction directly depends on methanol concentration [23], but invertase stability in methanol is the limiting factor [38]. Reaction between fructose and methanol is reversible with reaction equilibrium in the direction favoring hydrolysis of MF. Hence, the yield of this reaction is below 3% [23]. The kinetics of MF formation is interplay between formation and hydrolysis, according to reaction 2, see Fig. 2a. Initially the concentration of MF increases,

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but after 45 min it starts to decrease due to enzyme catalysed hydrolysis. The hydrolysis reaction is slower [23] and it possibly increases as the concentration of MF also increases. The highest amount of MF can be obtained when enzyme glycoform EINV1 is used. This is a consequence of the high stability of glycoform EINV1 in 30% v/v methanol in comparison to the other two glycoforms, namely EINV2 and EINV3. Also, the sample of total external invertase, EINV, provides lower yield than EINV1. This is because EINV is mixture that contains around 70% of EINV2 and EINV3, and only around 30% is EINV1, see Fig. 2b. On the other hand, the concentration of MF after 90 min of enzymatic reaction is lowest when enzyme glycoform EINV1 is used, see Fig. 6a. This can be a consequence of the high stability of glycoform EINV1 in 30% v/v methanol. Glycoform EINV1 retains a substantial amount of activity in these conditions after 90 min, Fig. 3, and therefore the hydrolytic reaction can proceed toward equilibrium catalyzed by EINV1. The hydrolytic enzymatic reaction with EINV2 and EINV3 could not proceed with the same intensity after 45 min, see Fig. 6a, since a substantial amount of the enzyme has lost its activity, Fig. 4. Hence, the total amount of MF after 90 min of enzymatic reaction is highest when the fraction EINV3 is used. Considering the kinetics of MF formation, the highest yield can be achieved with glycoform EINV1 after 45 min, see Fig. 6, in 30% v/v methanol, under the given reaction conditions. In order to benefit from this, a technological solution should be developed in order to stop the enzymatic reaction after 45 min, or by continuous removal of MF from the reaction mixture. As can be seen from Fig. 6b, applying EINV1 instead of preparation of total external invertase yield of MF can be improved for more than 50%. Moreover, increase of methanol concentration in reaction mixture from 20% to 30% increases conversion of sucrose to MF from 35% to  $44 \pm 5\%$ .

## 5 Concluding remarks

Baker's yeast external invertase is highly glycosylated, and the preparation of this enzyme is a mixture of different glycoforms (EINV). This mixture can be separated into glycoform fractions (EINV1–EINV3) by anion-exchange chromatography on monolithic support. Optimal subfractionation was achieved using a step elution gradient on QA-CIM discs. Differences in negative charge among glycoforms are a consequence of differently phosphorylated glycans. Individual glycoforms exhibit different stability in water-methanol solutions. The efficacy of enzymatic synthesis of methyl  $\beta$ -D-fructoside can be improved if a higher concentration of methanol is applied. The highest tolerated methanol concentration concerning invertase stability is 30% (v/v) under given conditions. Glycoform EINV1, that contains lowest amount of negative charged phosphate groups, exhibits significantly higher stability in 30% methanol than other glycoforms. Selection of this glycoform and its application in catalytic process improves yield of synthesis by more than 50%.

Chromatographic subfractionation enables control of glycosylation heterogeneity and isolation of individual glycoforms. This is of crucial importance for study of the role of different glycosylation forms in enzyme stabilization since production of individual glycoforms is still highly demanding task. Systematic studies of glycoforms and effect of glycans, their structure and position in protein sequence, on enzyme stability will provide information necessary to better understand glyco-code and provide basis for further improve of enzymatic-catalyzed industrial processes.

## Acknowledgements

*The authors acknowledge support from Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 172048 and 451-03-68/2020-14/200026). Programs of the European Union FP7 HTP-GlycoMet (contract No. 324400; author U. Andjelkovic and D. Josic) and FP7 RegPot FCUB ERA (GA No. 256716).*

## Conflict of interest

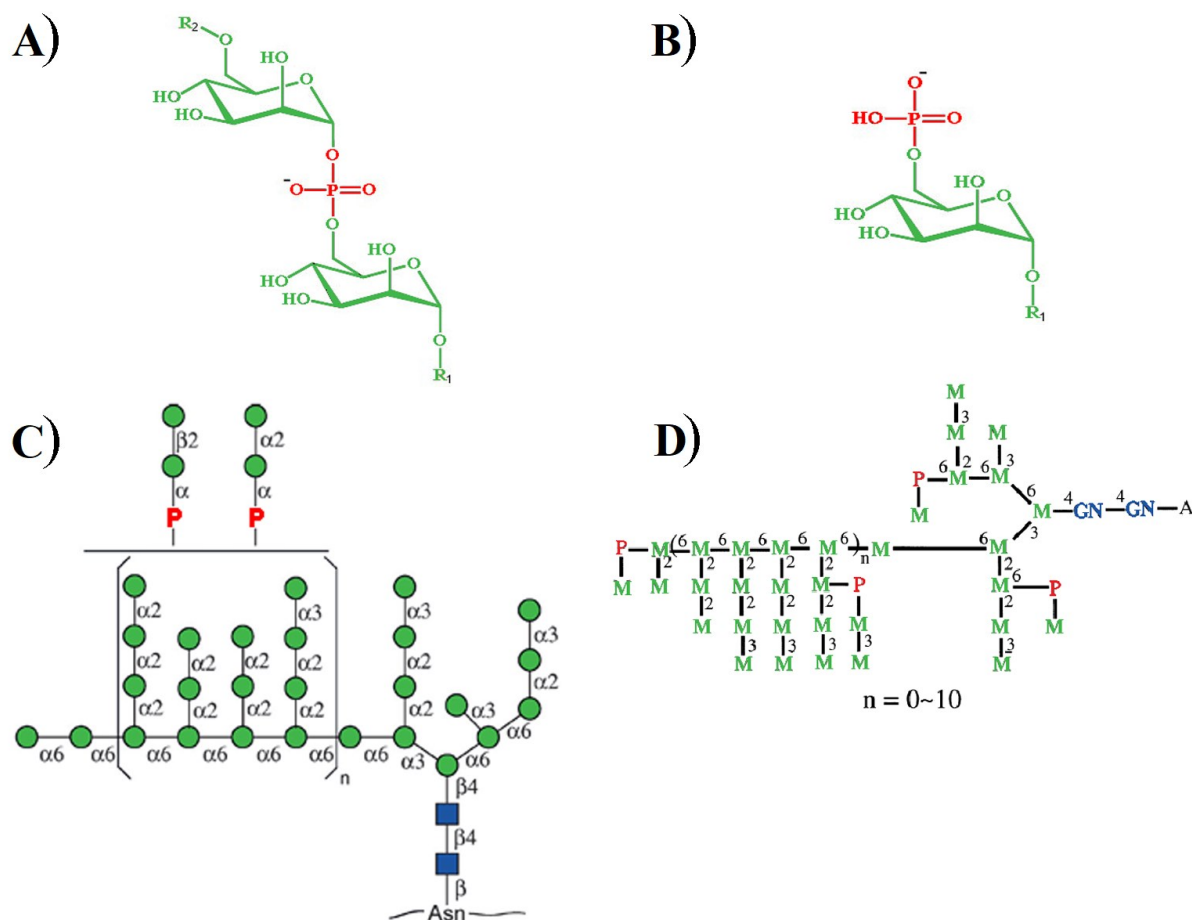
*The authors declare that they have no conflict of interest*

## 6 References

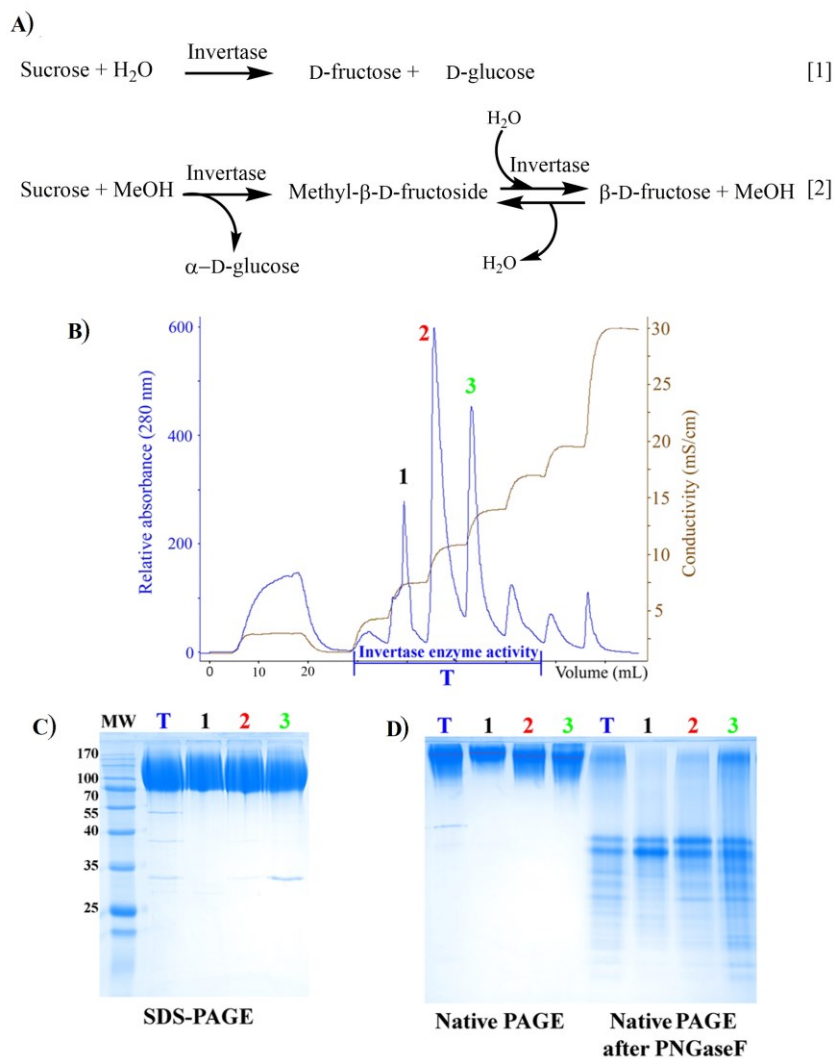
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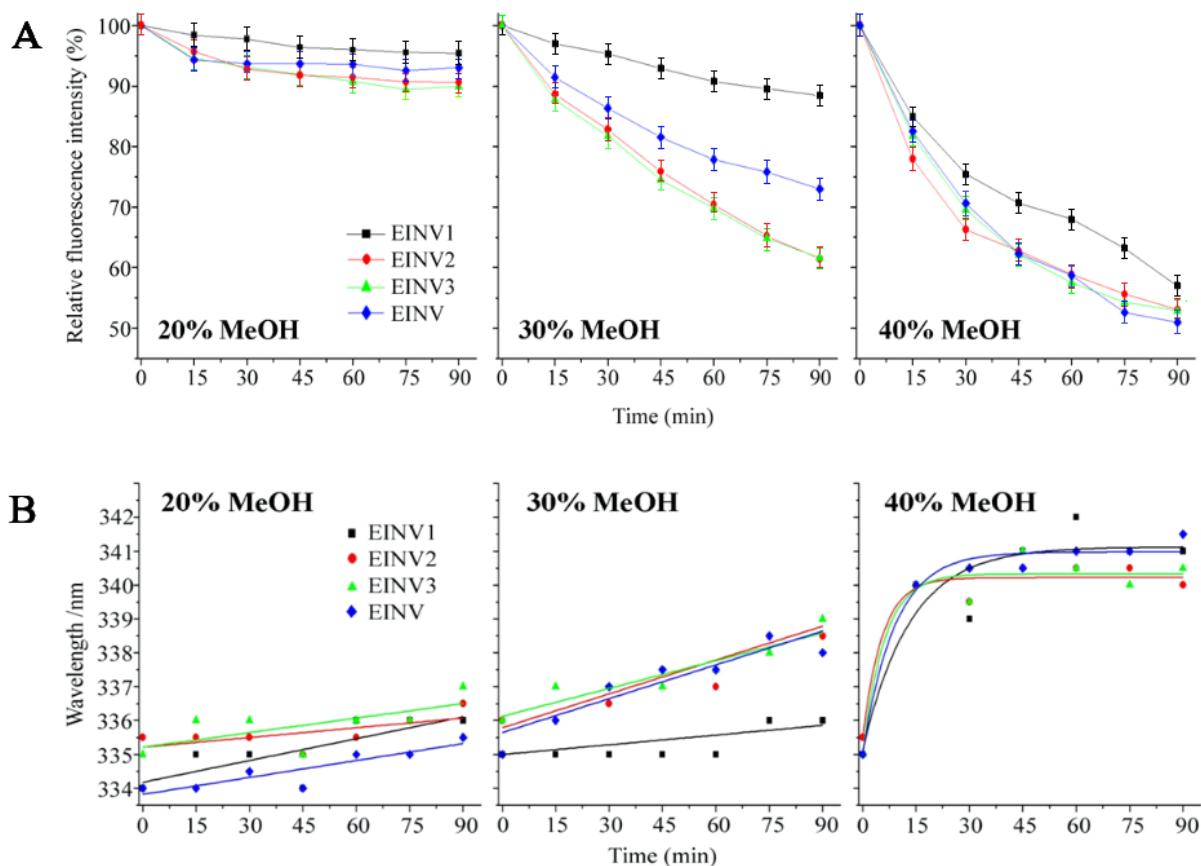


**Fig. 1:** a) Phosphate attached to mannose in the form of diester [26]; b) Phosphate attached to mannose in the form of monoester [28]; c) General structure of high mannose N-glycans of external invertase (reproduced with permission from [13]); d) General structure of high mannose N-glycans of external invertase (reproduced with permission from [27]).

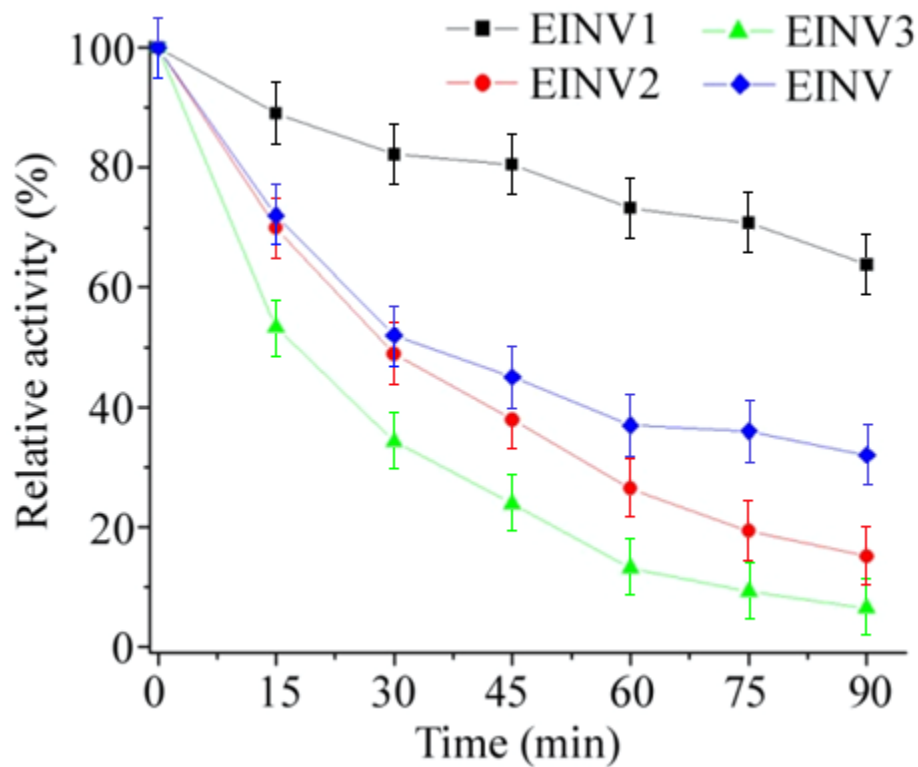


**Fig. 2:** a) Invertase catalyzed reaction between sucrose and water [1] or methanol [2]; b) Chromatogram shows separation of *S. cerevisiae* external invertase glycoform populations from a preparation of total external invertase (EINV) using step-gradient elution from anion-exchange CIM-QA disc (peak 1 – EINV1, peak 2 – EINV2, peak 3 – EINV3, the peaks before peak 1 and after peak 3 both contain invertase enzyme activity but are minor abundance glycoforms and are therefore not considered in this study); c) SDS-PAGE Electrophoretic profile of separated glycoforms; d) native-PAGE (red dots are added to show center of band) and native-PAGE electrophoretic profiles of separated glycoforms after deglycosylation with PNGaseF.

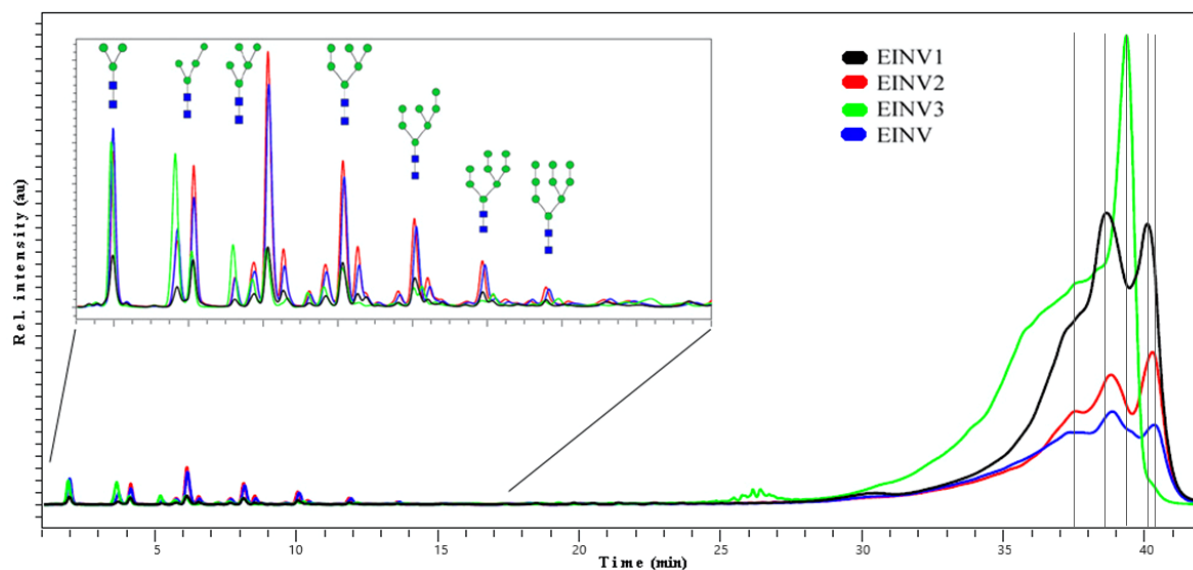




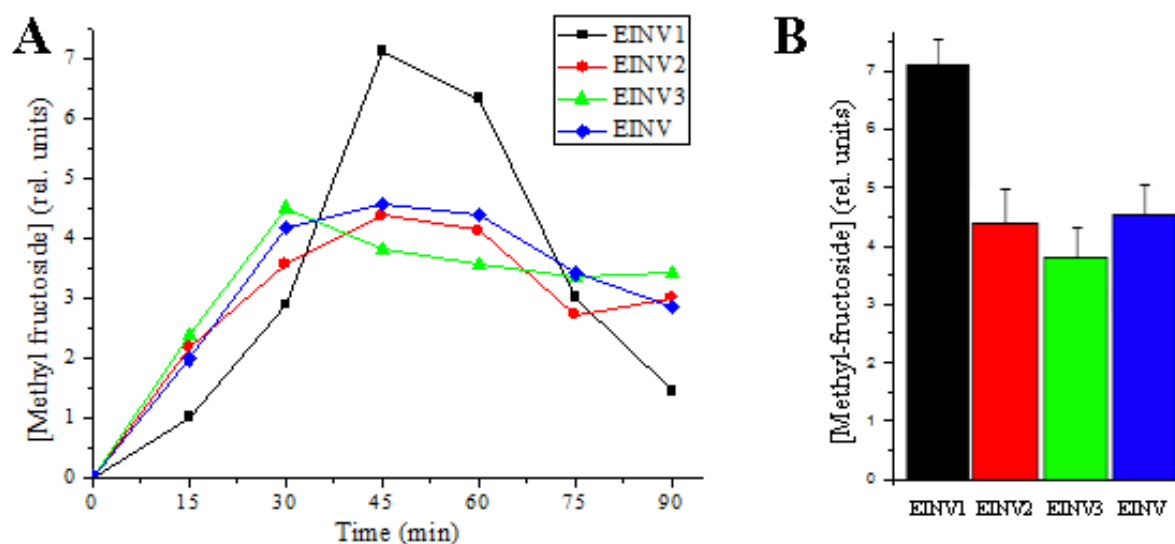
**Fig. 3:** Effect of different concentrations of methanol on external invertase glycoforms (EINV1–EINV3) and total external invertase (EINV) as monitored by fluorescent spectroscopy. Different concentrations (v/v) of methanol in 50 mM acetate buffer pH 5.0 at 40°C. (a) The change of intensity of intrinsic fluorescence at 340 nm. (b) The wavelength shift of the fluorescence emission maximum.



**Fig. 4:** Time courses of inactivation of external invertase glycoforms (EINV1–EINV3) and the total external invertase (EINV) with 30% (v/v) methanol in 50 mM acetate buffer pH 5.0 at 40°C.



**Fig. 5:** Chromatographic separation of N-glycans released by PNGaseF from external invertase glycoforms (EINV1–EINV3) and total external invertase (EINV1). Inset shows completely separated high mannose N-glycans with 3–9 mannose units. Right side of chromatogram shows separation of high mannose N-glycans that contain between 10 to >70 mannose units, grey lines are added to guide eyes to easier observe differences in chromatogram profile between glycoforms.



**Fig. 6:** Formation of methyl  $\beta$ -fructoside by external invertase glycoforms (EINV1–EINV3) and the total external invertase (EINV) in the presence of 30% (v/v) methanol, 0.3 M sucrose in 50 mM acetate buffer pH 5.0 at 40°C. (a) reaction between sucrose and water (1) or methanol (2); (b) Kinetic studies; (c) Relative concentrations of synthesized methyl  $\beta$ -fructoside after 45 min of enzymatic reaction.