Thermal Stability of Fructosyltransferase from *Aureobasidium pullulans**

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The thermal stability of a biocatalyst in the form of whole cells of the fungi *Aureobasidium pullulans* exhibiting fructosyltransferase activity was investigated during a batch catalytic process of fructooligosaccharide production. The stability of fructosyltransferase was monitored during 8 h with respect to temperature and overall saccharide concentration. A fructosyltransferase activity assay was developed for the monitoring of enzyme activity that was based on the rate of formation of 1-kestose measured by an HPLC method. It was found that the biocatalyst inactivated rather quickly at temperatures above 60 °C even at high saccharide concentrations. The stabilizing effect of saccharide concentration on the fructosyltransferase activity was investigated at 55 °C. A kinetic equation of enzyme inactivation, based on a reversible one-step reaction and simple exponential relationships of the rate constants and on the overall saccharide concentration, was suggested and statistically verified.

Fructosyltransferase (EC 2.4.1.9.; FTase) catalyzes the transfer of fructosyl moiety to the fructosyl part of sucrose or a fructooligosaccharide (FOS) whereby an oligomer by one fructosyl unit larger is formed. FTases from fungal species such as *Aureobasidium pullulans, Aspergillus niger, Aspergillus japonicus* or *Aspergillus sydowi* preferably produce 1^F-type FOS's which consist of short linear chains of D-fructose units linked by (2→1)-β-glycosidic bonds and carry a single D-glucose unit at the nonreducing end of the chain linked by (1→2)-α-glucosidic bond as in sucrose [1—4]. Typical oligomers obtained by the catalytic action of fungal FTases are 1-kestose, nystose, and 1^F-β-fructofuranosylnystose the molecules of which contain 2—4 fructosyl units. Various aspects of FOS production and applications have been reviewed in [1, 5].

A long-term process stability of an enzyme preparation is a common prerequisite of successful large-scale operation of a biocatalytic process. It is therefore not surprising that several studies have dealt with the stability of FTase [6—9]. Some of them were made in buffer solutions in the absence of substrate [6, 7] whereas high sucrose concentrations were applied in the others [8, 9]. Sucrose concentrations from 600 to 850 g dm^{-3} are used in the industrial production of FOS's, besides other means, to stabilize the enzyme and prevent its fast inactivation at temperatures of about 55 °C [1].

The objective of this study was to investigate the thermal stability of a FTase in the form of whole cells of *Aureobasidium pullulans* CCY 27-1-94, which was selected and optimized in a previous study as a biocatalyst providing the highest yield of FOS's [10]. In the first step, a method of FTase activity determination had to be developed. The focus of the investigation of the biocatalyst stability was the determination of the kinetics of FTase inactivation including the influence of saccharide concentration. This knowledge is important for the elaboration of the fundamental kinetics of FTase action for which measurements under conditions of simultaneous enzyme inactivation are needed.

EXPERIMENTAL

Biocatalyst Preparation

Whole cells of *Aureobasidium pullulans* CCY 27-1-94 were used in this study as a biocatalyst possessing FTase activity. They were cultivated at the conditions reported in our previous work [10]. The fermentation medium contained in g dm^{-3}: sucrose – 200, yeast extract – 10, $MgSO_4 \cdot 7H_2O$ – 0.5, K_2HPO_4 – 5, and $NaNO_3$ – 10. The pH was set to 6.5. Two portions of 100 cm^3 of fermentation medium were transferred into

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500 cm^3 Erlenmeyer flasks and the cultivation ran for 144 h at 28 °C and a rotational shaking frequency of 180 min^{-1}. The cells formed were both in mono- and multicellular forms and their colour was pink. After cultivation, the cells were washed with physiological solution several times and the physiological solution was removed by filtration. The cells were stored in the freezer.

The total mass of the wet cells obtained was 10.1 g. The dry matter content of the cells, determined gravimetrically, was 31.3 mass %. The activity of whole cells was determined using the method developed in this study and it was found to be 232 U per gram of dry matter that is 3.7 U cm^{-3} of fermentation medium.

Analysis of Sugars

HPLC analyses were performed on an equipment consisting of a Maxi-Star K-1000 pump, an on-line degasser, an injection valve, a differential refractive index detector, all from Knauer (Berlin, Germany), and a column thermostat Jetstream Plus II (Thermotechnic Products, Germany). The refractive index detector was operated at 32 °C. Chromatographic data were recorded in the form of the dependence of the refractive index difference, Δn_D, on the retention time, t_R, and evaluated using a CSW chromatographic station (DataApex, Prague, Czech Republic).

Before injection, the samples were filtered through a 0.2 μm filter and diluted with water. Anal. grade 1-kestose, nystose, and 1^F-fructofuranosylnystose (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and sucrose, glucose, and fructose (Sigma, Deisenhofen, Germany), respectively, were used as standards.

Ion-Exclusion Chromatography

Two HPLC columns were used in ion-exclusion mode, EuroKat Pb (Knauer, Berlin, Germany) and Rezex RSO-Oligosaccharide (Torrance, CA, USA). The columns were filled with a sulfonated polystyrene —divinylbenzene copolymer resin in the appropriate cationic form. The mobile phase was double distilled water. Other specifications of the columns and conditions of analysis were as follows:

– EuroKat Pb: Pb^{2+} form, 300 mm × 8 mm i.d., particle size 10 μm, 8 % of resin cross-linking, flow-rate 0.8 cm^3 min^{-1}, column temperature 80 °C;

– Rezex RSO: Ag^+ form, 200 mm × 10 mm i.d., particle size 8 μm, 4 % of resin cross-linking, flow-rate 0.2 cm^3 min^{-1}, column temperature 40 °C [11].

Reversed Phase Chromatography

The reversed phase column was Nucleosil 120-10 C18 (Macherey-Nagel, Duren, Germany) filled with octadecylsilane-modified silica. The column dimensions were 250 mm × 8 mm i.d., particle size 10 μm. The mobile phase was double distilled water at the flow-rate of 2 cm^3 min^{-1}. The column temperature was set to 25 °C.

Enzyme Activity Assay

One unit of FTase activity was defined as the amount of enzyme that produces 1 μmol of kestose per minute at standard conditions (substrate concentration 700 g dm^{-3} of sucrose in 0.1 M phosphate buffer, temperature 55 °C, pH 5.5) which were found optimal in our previous study [10]. The mass ratio of cell suspension and sucrose solution was 1 : 9 and the overall volume of the reaction mixture was about 10 cm^3. The activity measurement was made in a mechanically stirred and thermostated batch reactor for 1 h. Every 10 min, a sample was taken and boiled for two minutes to stop the reaction. The concentration of 1-kestose was determined by HPLC on a EuroKat Pb column. The enzyme activity was determined from the slope of the time dependence of 1-kestose concentration.

Kinetic Measurement

The kinetic measurements of the inactivation of whole-cell FTase from *A. pullulans* were performed for 8 h in the reactors of the same type as those used for the activity assay. Also the reaction mixture volume, cell-to-sucrose solution mass ratio, and pH were the same. The influence of temperature on the FTase stability was studied in the interval of 55—65 °C and at the saccharide concentration of 700 g dm^{-3}. For the measurement at 55 °C, however, the initial sucrose concentration was varied in the interval from 0 to 700 g dm^{-3}. The residual activity of FTase was determined every two hours with the volume of 1 cm^3 samples.

Mathematical Modelling

The inactivation of FTase was assumed to proceed according to a simple mechanism of a single-step reversible transformation of the active form E of the enzyme into an inactive form D

$$\mathrm{E} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{D} \qquad (A)$$

The kinetic rate equation derived from the above mechanism had the form

$$r = k_{+1}c_E - k_{-1}c_D \qquad (1)$$

where r is the reaction rate, k_{+1} and k_{-1} are the rate constants of forward and backward reactions, and c_E and c_D are the molar concentrations of active and inactive enzyme forms, respectively.

The material balances of active and inactive forms

were expressed by the following differential equations

$$\frac{\mathrm{d}c_\mathrm{E}}{\mathrm{d}t} = -r \qquad (2a)$$

$$\frac{\mathrm{d}c_\mathrm{D}}{\mathrm{d}t} = r \qquad (2b)$$

and initial conditions

$$t = 0 \qquad c_\mathrm{E} = c_{\mathrm{E}0} \qquad c_\mathrm{D} = 0 \qquad (2c)$$

It was therewith assumed that all enzyme was initially in the active form with the initial concentration of $c_{\mathrm{E}0}$.

A convenient transformation of equations (*2a*—*2c*) was achieved by introducing dimensionless concentrations of the active and inactive enzyme forms C_E and C_D

$$C_\mathrm{E} = \frac{c_\mathrm{E}}{c_{\mathrm{E}0}} \qquad (3a)$$

$$C_\mathrm{D} = \frac{c_\mathrm{D}}{c_{\mathrm{E}0}} \qquad (3b)$$

The mathematical model was thus formed by the following equations

$$\frac{\mathrm{d}C_\mathrm{E}}{\mathrm{d}t} = -k_{+1}C_\mathrm{E} + k_{-1}C_\mathrm{D} \qquad (4a)$$

$$\frac{\mathrm{d}C_\mathrm{D}}{\mathrm{d}t} = k_{+1}C_\mathrm{E} - k_{-1}C_\mathrm{D} \qquad (4b)$$

$$t = 0 \qquad C_\mathrm{E} = 1 \qquad C_\mathrm{D} = 0 \qquad (4c)$$

Eqns (*4a*—*4c*) show that the model was independent of absolute values of the enzyme concentration which were slightly different in the individual experiments. The influence of saccharide concentration on the rate constants was expressed by the following exponential relationships

$$k_{+1} = k^0_{+1} \exp\left(-\frac{\rho_\mathrm{S}}{\rho_{+1\mathrm{S}}}\right) \qquad (5a)$$

$$k_{-1} = k^0_{-1} \exp\left(\frac{\rho_\mathrm{S}}{\rho_{-1\mathrm{S}}}\right) \qquad (5b)$$

where ρ_S is the overall mass concentration of saccharides and the parameters k^0_{+1} and k^0_{-1} represent the rate constants of forward and backward reactions in the absence of saccharide. $\rho_{+1\mathrm{S}}$ and $\rho_{-1\mathrm{S}}$ are the saccharide concentration characteristic constants that correspond to the saccharide mass concentration values at which $k_{+1}/k^0_{+1} = 1/\mathrm{e}$ or $k_{-1}/k^0_{-1} = \mathrm{e}$, respectively.

The parameters of the model were estimated by the simultaneous fit of data obtained at different saccharide concentrations using a parameter estimation software Athena Visual Workbench (Stewart & Associates Engineering Software, Madison, WI, USA).

RESULTS AND DISCUSSION

A convenient method for the determination of FTase activity is a prerequisite for the stability study. In developing such a method, one has to take into account the complexity of the reaction scheme in this catalytic process. The process of formation of FOS's is represented by the sequence of reactions of subsequent fructosylation of sucrose. Fig. 1 shows that at high conversions of sucrose, the reaction mixture contained a spectrum of oligomers with the degree of polymerization ranging from 3 to 6. The presence of a fructose peak in this chromatogram indicates that the biocatalyst possesses the hydrolytic activity besides the transferase activity. Fortunately, the spectrum of reaction products is not so rich in the early stage of the process. The fructosyl moiety is transferred here to a sucrose molecule and a trimer is formed as the main product and glucose as a by-product. This is illustrated by the chromatogram in Fig. 2 of a reaction mixture at about 2 % conversion of sucrose.

Another aspect, which should be dealt with in the case of fungal FTases, is the regiospecificity of the binding of the fructosyl moiety to sucrose. Depending on the enzyme source, the trimers produced can be 1-kestose, 6-kestose, neokestose or isokestose [1—4, 12]. By comparing the retention times of the chromatographic peaks in Figs. 1 and 2 with those of standards,

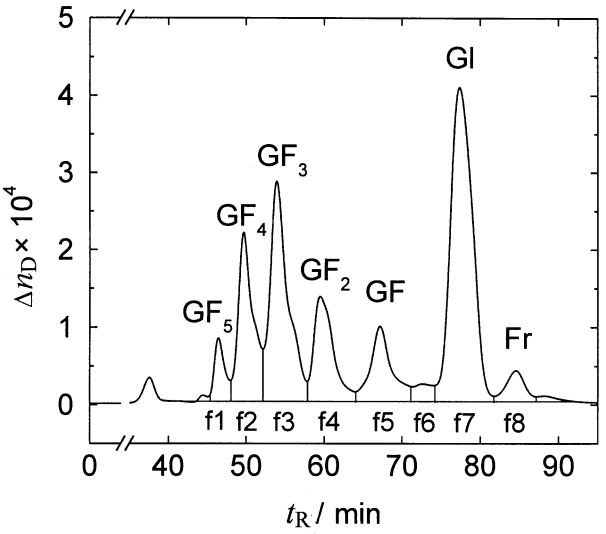

Fig. 1. Semipreparative chromatographic separation of the mixture of FOS's, sucrose, glucose, and fructose on the column Rezex RSO. The sample was prepared by action of whole cells of *A. pullulans* on sucrose during 8 h (initial sucrose concentration of 700 g dm^{-3}, temperature 55 °C, pH 5.5, mass ratio of the wet cells and substrate 1 : 9). Collected fractions were marked as f1—f8. Peaks: Gl – glucose, Fr – fructose, GF – sucrose, GF_n – fructooligosaccharides constituted from one glucopyranosyl and n fructofuranosyl moieties, n = 2—5.

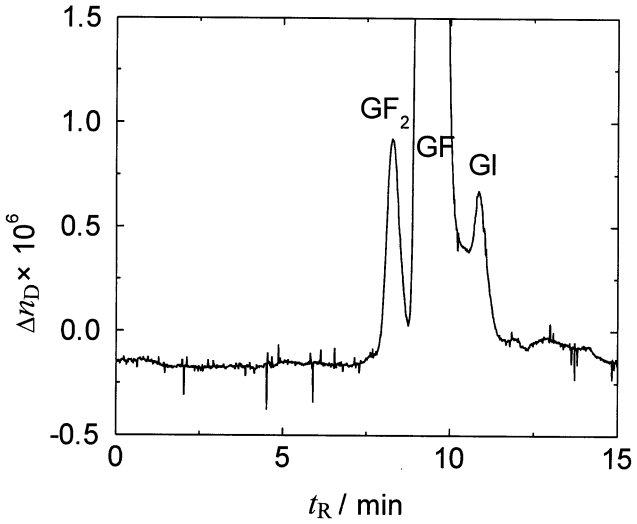

Fig. 2. Chromatogram of the mixture of 1-kestose, sucrose, and glucose on the column EuroKat Pb. The sample is a reaction mixture at about 2 % conversion of sucrose. Peak labels as in Fig. 1.

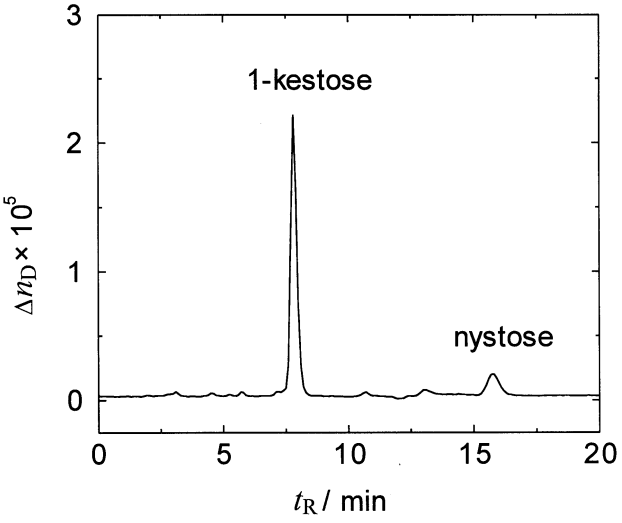

Fig. 3. Analysis of the fraction f4 from the separation presented in Fig. 1 on the column Nucleosil C18.

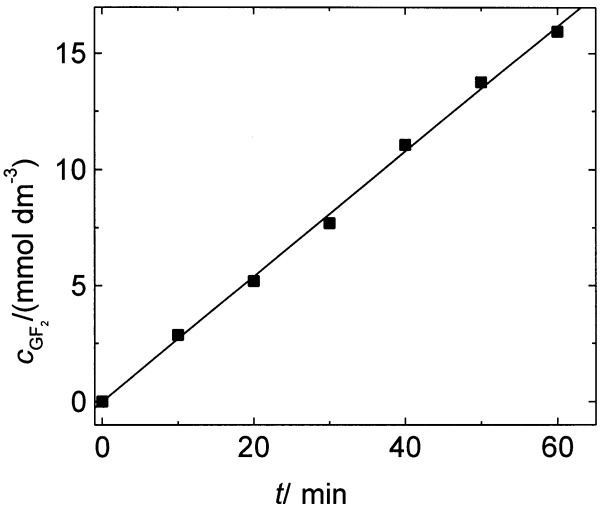

Fig. 4. Example of FTase activity determination by the initial reaction rate method. The best linear fit of the dependence of 1-kestose concentration c_{GF_2} on time t was $\{c_{GF_2}\} = 0.270\,\{t\} - 0.002$, with the regression coefficient of 0.9992. The solid line represents the calculated course of 1-kestose concentration. The enzyme activity was thus 270 U dm^{-3}.

the trimer peak could be assigned as 1-kestose. We decided to confirm this conclusion by the analysis of the trimer fraction f4 obtained from the semipreparative ion-exclusion column (Fig. 1) on a reversed phase column. *Praznik* and *Spies* [13] demonstrated that this type of column enables a very good separation of different structural isomers of FOS's. The analysis of the fraction f4 showed that this fraction contained only two compounds, 1-kestose and nystose (Fig. 3) where the small portion of nystose arose mainly from an imperfect collection of the fractions. This result unambiguously confirmed that the FTase from *A. pullulans* had a very high regiospecificity for the transfer of fructosyl residues to the OH-1^F position of sucrose.

Based on the results presented above, we decided to define the FTase activity as the rate of production of 1-kestose obtained in a batch measurement of the so-called initial reaction rate. The application of the initial reaction rate method requires a low substrate conversion and linear dependence of the concentration of the monitored species on time. Applying the conditions described in Experimental, 1 h was set as the overall time of measurement. This time was sufficient to achieve a good signal-to-noise ratio in the HPLC analysis of 1-kestose. The sucrose conversion after 1 h was less than 2 % in this experiment as well as in other cases. Fig. 4 illustrates a measurement of FTase activity where the value 270 U dm^{-3} was estimated directly from the slope of the time course of the 1-kestose concentration.

Our previous investigation of FOS's production showed that, in the temperature interval of 55 to 65 °C, the total yield of FOS's at the initial sucrose concentration of 700 g dm^{-3} and pH 5.5 was essentially temperature-independent although the reaction rates increased with temperature [10]. This could be caused by inactivation of FTase and therefore the investigation of the temperature influence on the inactivation of the enzyme was made in this work. The enzymatic production of FOS's was carried out under the same conditions as in the previous study [10], and the reaction mixture was periodically sampled for the determination of FTase activity. A significant decrease of enzyme activity was observed at 65 °C (Fig. 5) when the value after 8 h was 57 % of the initial activity. At 60 °C and 55 °C, the decrease of enzyme activity was much slower and the residual activity at the end of

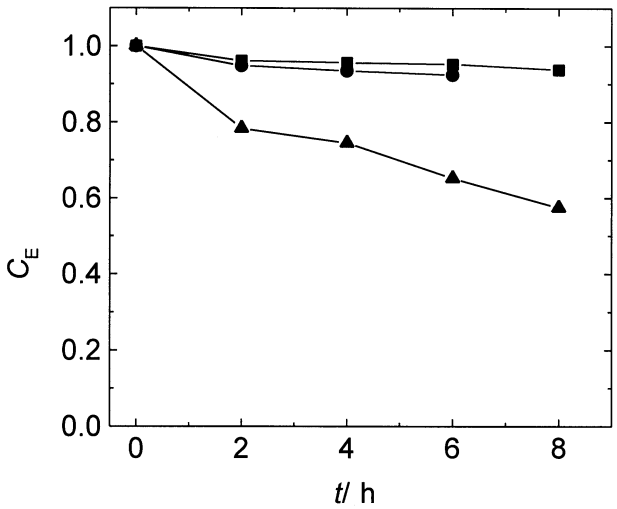

Fig. 5. The influence of temperature on the loss of FTase activity during a batch enzymatic reaction at the initial sucrose concentration of 700 g dm^{-3}. ■ 55 °C, ● 60 °C, ▲ 65 °C.

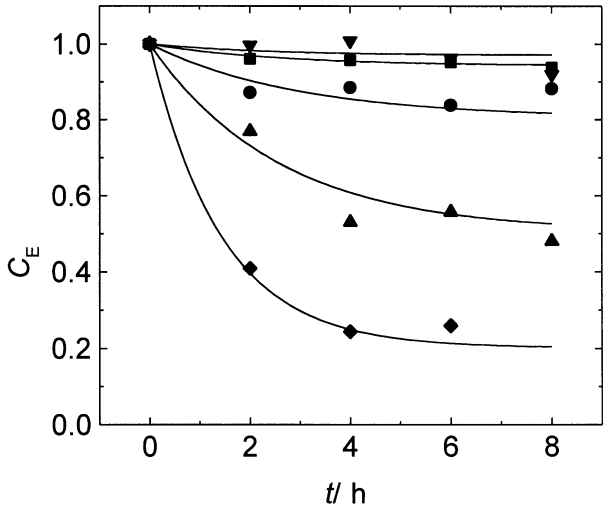

Fig. 6. The influence of the overall saccharide concentration on the inactivation of FTase during a batch enzymatic reaction at 55 °C. The solid lines were computed from the model equations using the estimated parameter values. Overall saccharide concentration: 0 g dm^{-3} (♦), 200 g dm^{-3} (▲), 400 g dm^{-3} (●), 600 g dm^{-3} (▼), 700 g dm^{-3} (■).

experiment was about 90 %.

Similar experiments were designed for the investigation of the influence of the overall concentration of saccharides on the FTase stability. The initial sucrose concentrations were varied here from the typical process value of 700 g dm^{-3} towards lower values. One experiment was done without reaction by incubating the biocatalyst in buffer solution. The influence of saccharides on the stability was investigated at 55 °C and the results of the measurements of enzyme activity during 8 h of the batch enzymatic reaction are presented in Fig. 6. The results clearly show a stabilizing effect of high concentrations of saccharides on the FTase activity. The rate of inactivation of enzyme was relatively slow at the overall saccharide concentration above 600 g dm^{-3} but it quickly grew below this threshold. As expected, the rate of inactivation was the fastest in buffer solution where the activity of enzyme dropped to about one quarter of its initial value already within 6 h.

As has been mentioned above, it would be useful to obtain an equation that would describe the loss of FTase activity with respect to time and initial sucrose concentration. A preliminary quantitative analysis of the results presented in Fig. 6 showed that any single curve of the activity loss with time could be described using a kinetic equation derived from a simple reversible mechanism. This is evident also from the shape of the inactivation curves where the initial exponential phase is followed by a phase of a much slower change of activity which could be approximated as a phase of apparent equilibrium. The rate constants obtained in this way exhibited dependences on the overall saccharide concentration, which could be expressed through simple exponential functions. The model, described in detail in Experimental, was then applied to a simultaneous fit of all data presented in Fig. 6.

Using the parameter estimation software Athena Visual Workbench, a good fit of the experimental data with the model was obtained in which the residual sum of squares of relative activity values was 0.025 which represented an error of the approximated activities of 3.5 % related to the initial activity values. The activity profiles evaluated from the model are plotted as solid lines in Fig. 6. The estimated parameter values of the model are given in Table 1. The values of the rate constants of the reversible mechanism of inactivation calculated from these parameters are presented in Fig. 7 as a function of the overall saccharide concentration. Fig. 7 clearly demonstrates a strong stabilizing influence of saccharides on FTase.

Table 1. The Parameters of the Model of FTase Inactivation

Parameter	Estimated value ± standard deviation
k^0_{+1}/min^{-1}	0.58 ± 0.08
k^0_{-1}/min^{-1}	0.15 ± 0.04
ρ_{+1S}/(g dm^{-3})	190 ± 30
ρ_{-1S}/(g dm^{-3})	660 ± 570

In the saccharide-free solution, the rate constant of the forward denaturation reaction, k_{+1}, was about four times larger than the rate constant of the backward renaturation reaction, k_{-1}. This means that the inactive enzyme form is thermodynamically favourable at very low sugar concentrations. At the overall saccharide concentration of 200 g dm^{-3}, how-

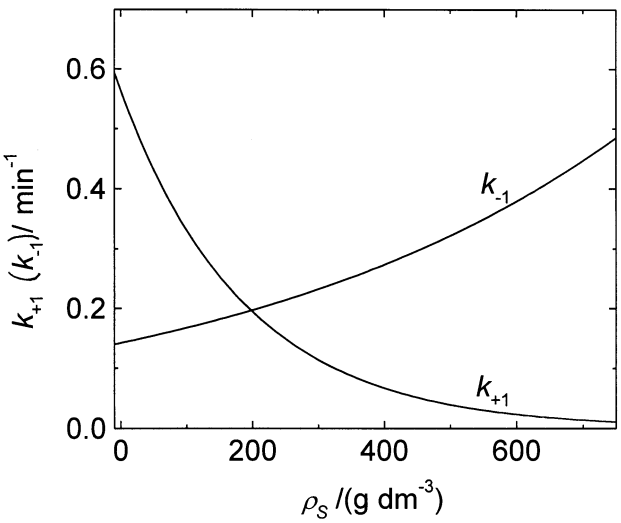

Fig. 7. The dependence of the rate constants of the reversible mechanism of enzyme inactivation on the overall saccharide concentration calculated from the model.

ever, the rate constants were already equal, which indicated that the enzyme activity should level approximately on the value of 50 % of the initial activity. The native enzyme is a preferable thermodynamic state at the concentrations above 200 g dm^{-3} when, at the highest investigated concentration of 700 g dm^{-3}, the rate constant of renaturation was two orders of magnitude larger than the denaturation rate constant. This explains the very low decrease of FTase activity at the high saccharide concentrations presented in Fig. 6.

Table 1 further shows that the relative standard deviations of individual parameters were quite different. Both parameters related to the backward renaturation reaction, were estimated with larger relative errors than the parameters of the forward denaturation reaction. This is understandable since the model assumes that the value of the initial concentration of the inactive form is zero. The initial rate of the activity loss thus represents also the initial rate of the forward reaction. The backward reaction is, in general, more pronounced at the later phases of the process. The later phases were however not achieved in the cases of high saccharide concentrations as follows from Fig. 6. The values of the rate constants of the backward reaction at high concentrations are then loaded with larger uncertainties. These uncertainties are responsible for the larger value of standard deviation of the concentration constant ρ_{-1S}.

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