The inulin hydrolysis by recombinant exo-inulinases: determination the optimum temperatures and activation energies

Justyna Miłek¹

Received: 8 February 2021 / Accepted: 19 September 2021 / Published online: 9 October 2021
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Abstract

The advantages of recombinant enzymes over native include the control in a production environment, product purity and also high yield. The paper presents the determination the optimum temperatures and the activation energies for various origin recombinant exo-inulinases, among others from *Aspergillus niger*, *A. awamori*, *Kluyveromyces marxianus* and *K. cicerisporus*. The parameters were estimated based on the literature of the activity curves versus temperature for hydrolysis of inulin. It was assumed that both the hydrolysis reaction process and the deactivation process of recombinant exo-inulinase were first-order reactions by the enzyme concentration. A mathematical model describing the effect of temperature on recombinant exo-inulinase activity was used. Based on the comparison analysis, values of the activation energies E_a were in the range from 32.01 ± 7.80 to 43.83 ± 4.87 kJ mol⁻¹, the deactivation energies E_d were in the range from 83.93 ± 4.82 to 352.44 ± 14.26 kJ mol⁻¹ and the optimum temperature T_{opt} were obtained in the range from 318.91 ± 1.19 to 328.76 ± 0.25 K.

Keywords Optimum temperature · Recombinant exo-inulinase · Activation energy · Deactivation energy

Introduction

Enzymes are characterized, among others by the optimum temperature. When its exceeded, there is thermal denaturation of enzyme and a rapid decrease in enzyme activity. The knowledge of the optimum temperature T_{opt} and the activation energy E_a and deactivation energy E_d of the enzyme enables the optimization of the bioprocess and the reduction of its costs at the same time. The T_{opt} value is mostly determined experimentally. On the other hand, the value of the activation energy E_a and E_d can be determined from the curves of the dependence of the logarithm of the reaction rate ($\ln v$) on the reciprocal of temperature ($1/T$), the so-called Arrhenius dependence [1]. However, the determined values of E_a and E_d by application of the traditional method is burdened with an error [2, 3].

Inulinases (2,1- β -D-fructan fructanohydrolase) catalyze the hydrolysis of inulin to obtain fructose with a yield of

about 90–95%. Inulin consists of linear chains of 2,1- β -D-fructofuranose molecules terminated with a glucose residue at the reducing end. Inulin is a reserve carbohydrate (0.5–22%) in plant tubers like Jerusalem artichoke, chicory, garlic and onion, leek, rye, barley, dandelion, burdock, banana [4–7]. Inulinases can be divided into endo-inulinases (EC 3.2.1.153) and exo-inulinases (EC 3.2.1.80). Exo-inulinases remove terminal fructose residues from the non-reducing end of inulin, and endo-inulinases act on the internal bonds of the inulin molecule and can be used to produce fructooligosaccharides [8–10]. Inulinases occur in plants, fungi, yeasts and bacteria. Strains belonging to *Aspergillus* sp. and *Kluyveromyces* sp. are the most common and preferred for the production of inulinase. Inulinases obtained from them are characterized by high activity and high thermostability [5].

The optimum value of pH for inulinase *A. niger* is the same for both exo- and endo-inulinase and equals to 5.0. However, the optimal temperatures of most exo-inulinase are above 50 °C [7].

Inulinases can be used for industrial purposes in the production of high-fructose syrups, bioethanol production, production of fructose and glucose oligosaccharides (therapeutic products as prebiotics), unicellular oils and production of

✉ Justyna Miłek
jmilek@utp.edu.pl

¹ Department of Chemical and Biochemical Engineering,
Faculty of Chemical Technology and Engineering,
Bydgoszcz University of Science and Technology,
Seminarnyja 3, 85-326 Bydgoszcz, Poland

a unicellular protein in the production of citric acid, butanediol, alcohols and lactic acid (Table 1) [5, 11–15].

The advantages of recombinant enzymes over native include the control in a production environment, product purity and also high yield. Efficient production of inulinases on an industrial scale can significantly reduce the costs of biocatalyst, reduce energy consumption and improve the efficiency of fructose production [8].

Some exo-inulinase genes have been expressed in bacteria *Escherichia coli* and yeast *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Pichia pastoris* and fungi *Penicillium canescens* [14–20]. The goal of scientists is to obtain the recombinant inulinases that are more thermostable at different pH than the native strains.

There have been many publications in the literature on recombinant exo-inulinases from fungi *A. niger* [14, 17, 18, 20], *A. awamori* [15] and from yeast *K. cicerisporus* [16] and *K. marxianus* [19]. However, processes involving this recombinant exo-inulinase for industrial purposes cannot be designed and optimized without knowing the kinetic parameters of the process and thus the effect of temperature on activity recombinant exo-inulinase [21]. Hydrolysis with recombinant exo-inulinases is usually carried out at temperatures higher than 45 °C then, a significant inactivation of the enzyme may occur. Therefore, it is necessary to determine the optimum temperature T_{opt} , activation energy E_a and activation energy of the deactivation process E_d for recombinant exo-inulinases. The method of determining the parameters based on experimental data on the effect of temperature on the activity recombinant of exo-inulinases has been presented [14–20].

The effect of temperature on recombinant exo-inulinase activity

The value of the activation energy E_a and the deactivation energy E_d of inulinase can be determined using: the enzymatic reaction rate curve v , as the relationship between $\ln v$ versus temperature inverse T or the curve of inulinase activity a , as dependence between $\ln a$ relation to the inverse of temperature T . Activation energies or deactivation energies in hydrolysis reactions involving various types of inulinase [21–23] were determined. In this work, the basic equation

(mathematical model) of the presented method is the equation describing the change in the dimensionless activity of the enzyme a depending on the temperature T as follows

$$a = \frac{\exp\left(\frac{(T_{\text{opt}}-T)E_d\beta}{RTT_{\text{opt}}(\exp\beta-1)}\right)\left(1 - \exp\left(-\beta\exp\left(\frac{(T-T_{\text{opt}})E_d}{RTT_{\text{opt}}}\right)\right)\right)}{1 - \exp(-\beta)}, \quad (1)$$

where T_{opt} is the temperature at which recombinant exo-inulinase shows maximum activity (K), E_d is the activation energy of the deactivation process (J mol⁻¹), R is gas constant (8.314 J mol⁻¹ K⁻¹), T is temperature (K) and dimensionless parameter β determines the relationship

$$\beta = k_{d0}t_a \exp\left(-\frac{E_d}{RT_{\text{opt}}}\right), \quad (2)$$

where k_{d0} is a pre-exponential factor the kinetic constant of the deactivation process of recombinant exo-inulinase (min⁻¹) and t_a is an inulin hydrolysis time of recombinant exo-inulinases (min).

Knowing the value of the activation energy of the deactivation reaction E_d and the parameter β , the activation energy E_a is determined by equation

$$E_a = E_d - \frac{E_d\beta}{\exp\beta - 1}, \quad (3)$$

Equations from equal (1) to equal (3) were used to determine the kinetic parameters of p-nitrophenyl palmitate hydrolysis by lipases [24], starch hydrolysis by α -amylase from porcine pancreas [25] and inulin hydrolysis by exo-inulinases *A. niger* [26] or by endo-inulinase *Aspergillus niger* [27].

Based on Eq. (1), the kinetic parameters E_d and T_{opt} were determined by a nonlinear regression according to the Levenberg–Marquardt procedure [24–32], determining the minimum sum of squared errors defined by Eq. (4)

$$\text{SSE}(E_a, \beta, T_{\text{opt}}) = \sum_{i=0}^n \frac{1}{(a_{\text{exp}})_i^2} \left((a_{\text{exp}})_i - a_{\text{cal}}(E_a, \beta, T_i, T_{\text{opt}}) \right)^2, \quad (4)$$

where $(a_{\text{exp}})_i$ - recombinant exo-inulinase of various origins activity determined experimentally, $a_{\text{cal}}(E_a, \beta, T_i, T_{\text{opt}})$ - recombinant exo-inulinase of various origins activity calculated from Eq. (1).

Table 1 Products from origin inulin hydrolysed by recombinant exo-inulinase

Substrate	Source inulinase	Products	References
<i>Asparagus</i> inulin	<i>K. marxianus</i> YS-1	High fructose syrup	[5]
Jerusalem artichoke	<i>A. niger</i>	Lactic acid	[11]
Jerusalem artichoke	<i>K. cicerisporus</i>	Ethanol	[12]
Jerusalem artichoke	<i>K. marxianus</i> ATCC 8554	Ethanol	[13]
Inulin	Recombinant <i>A. niger</i>	Fructose syrup	[14]

Results and discussion

Experimental literature data [14–20] were analyzed for exo-inulinases from various yeast and bacteria. Table 2 shows the conditions for measuring insulin activity, such as pH and a buffer type, time measurement. In most cases, the analyzed inulinase activities were determined by using the Nelson-Somogyi [15, 20] method and the DNS method [14–17], using dinitrosalicylic acid. The initial inulin concentration measured was in the range of 1%–5%.

Used strains by Chen et al. [14] has been obtained from own the Laboratory of Biology and Pharmaceutical Engineering, Wuhan Polytechnic University in China. *A. niger* exo-inulinase gene was codon-optimized and co-expressed with the protein in *Pichia* cells. In the hydrolysis of inulin by recombinant exo-inulinase *A. niger*, a fructose syrup product with a content of fructose of 95% was obtained.

Volkov et al. [15] analyzed the recombinant exo-inulinase obtained from exo-inulinase gene *A. awamori* strain into *P. canescens* A3. Used strains has been obtained from the Laboratory of Enzyme Biotechnology in Bach Institute of Biochemistry, Russian Academy of Sciences.

Gene encoding an exoinulinase was cloned from *K. cicerisporus* CBS4857 and expressed in *P. pastoris* X-33 [16]. Arjomand et al. [18] applied *A. niger* 5012 to the cloning process to *E. coli* DH5. The cloning process creates a mutant called Δ 6SL. The recombinant exo-inulinase *A. niger* 5012 Δ 6SL was lowering the activation energy of the catalysis and effective improving the catalytic performance. The purified by Zhang et al. [19] recombinant exo-inulinase *K. marxianus* CBS 6556 to *P. pastoris* showed an activity which is over 12-fold higher than those of other inulinases described previously. Liu et al. [20] used *K. marxianus* KM-0 strain (collection number 2E001023) and *Y. lipolytica* Po1h strain (collection number 2E0189) from Marine Microorganisms Culture Collection of China (MCCC).

The effect of temperature on the recombinant exo-inulinases activity

Based on experimental data on the change in the activity of recombinant exo-inulinases [14–20], and Eq. (1); the value of deactivation energies E_d , β parameters and temperatures optimal T_{opt} were determined from Eq. (1). Figures 1–7 show experimental data on recombinant exo-inulinases activity, along with activity curves plotted based on Eq. (1) for the values of the specified parameters E_d , E_a , β listed in Table 3.

Statistical analysis was used to analyze, interpret and summarize experimental data. Results expressed as mean values with error bars indicating standard deviations, whereas the 95% confidence band was additionally marked for the obtained curves. Data obtained from results were analyzed by nonlinear regression using Sigma Plot 12.3.

With the values of the activation energy parameter for the deactivation reaction E_d and the parameter β , the activation energy value was calculated based on Eq. (3).

Fig. 1 Activity of recombinant exo-inulinase *A. niger* CBS 513.8 by measurements Chen et al. [14]

Table 2 Conditions for measuring of recombinant exo-inulinases activity

pH buffer	t/min	Concentration of inulin/%	Source of inulinase	References
5.5 sodium acetate	5	5	<i>A. niger</i> CBS 513.8 to <i>P. pastoris</i>	[14]
5.0 sodium acetate	5	1	<i>A. awamori</i> to <i>P. canescens</i> A3	[15]
4.5 sodium acetate	10	2	<i>K. cicerisporus</i> CBS4857 to <i>P. pastoris</i> X-33	[16]
4.5 sodium acetate	5	1	Recombinant <i>A. niger</i> (Megazyme)	[17]
5.0 sodium acetate	8	1	<i>A. niger</i> 5012 to <i>E. coli</i> DH5 (<i>A. niger</i> 5012 Δ 6SL)	[18]
4.6 sodium acetate	30	5	<i>K. marxianus</i> CBS 6556 to <i>P. pastoris</i>	[19]
4.5 phosphate	10	2	<i>K. marxianus</i> KM-0 to <i>Y. lipolytica</i> Po1h	[20]

Fig. 2 Activity of recombinant exo-inulinase *A. awamori* by measurements Volkov et al. [15]

Fig. 5 Activity of recombinant exo-inulinase *A. niger* 5012 Δ 6SL by measurements Arjomand et al. [18]

Fig. 3 Activity of recombinant exo-inulinase *K. cicerisporus* CBS4857 by measurements Ma et al. [16]

Fig. 6 Activity of recombinant exo-inulinase *K. marxianus* CBS 6556 by measurements Zhang et al. [19]

Fig. 4 Activity of recombinant exo-inulinase *A. niger* by measurements Megazyme [17]

Fig. 7 Activity of recombinant exo-inulinase *K. marxianus* KM-0 by measurements Liu et al. [20]

Table 3 Values of parameters estimated recombinant exo-inulinases from various origin

Fig.	T_{opt}/K	β	$E_d/\text{kJ mol}^{-1}$	$E_a/\text{kJ mol}^{-1}$	E_d/E_a	References
1	318.91 ± 1.19	0.66 ± 0.11	120.87 ± 6.18	35.53 ± 7.17	3.40	[14]
2	321.60 ± 0.76	0.48 ± 0.09	144.92 ± 12.15	32.01 ± 7.80	4.53	[15]
3	323.00 ± 1.47	0.57 ± 0.16	126.18 ± 9.99	32.56 ± 10.35	3.86	[16]
4	324.32 ± 2.37	1.09 ± 0.26	83.93 ± 4.82	37.59 ± 9.23	2.23	[17]
5	326.84 ± 1.15	0.47 ± 0.14	149.40 ± 19.30	32.37 ± 12.08	4.62	[18]
6	328.39 ± 1.66	0.52 ± 0.12	176.91 ± 19.93	42.03 ± 12.72	4.21	[19]
7	328.76 ± 0.25	0.26 ± 0.02	352.44 ± 14.26	43.83 ± 4.87	8.01	[20]

The obtained calculation values E_a results are presented in Table 3.

The obtained parameters for recombinant exo-inulinase were presented in Table 3, according to the increasing value of the activation energy of the deactivation reaction.

Table 4 presents statistical data obtained while determining the parameters of recombinant exo-inulinases from various origin.

High regression coefficients R^2 above 0.926, standard errors of SSE estimation below 0.2113 were obtained; while statistical variability of E_d and T_{opt} parameters in most of the analyzed cases $p < 0.0001$. F -Fisher test values were from 31.33 to 1140.12 with a probability value P in most of the analyzed cases is low 0.0003 confirmed that it was appropriated to apply Eq. (1) when determining parameters. Also, Figs. 1–7 present standard deviation errors for experimental points, while the 95% confidence bonds were marked for the obtained curves.

Optimum temperature T_{opt}

The estimated average values of the optimum temperatures T_{opt} for the experimental data [14–20] were found in the range from 318.91 ± 1.19 to 328.76 ± 0.25 K. Table 3 shows that the highest temperature value was obtained for measurements of activity for recombinant exo-inulinase *K. marxianus* KM-0 to *Y. lipolytica* Po1h [20].

For the *A. niger* no-recombinant exo-inulinase [26], it was found that they were in the range from 325.25 ± 0.41

to 337.35 ± 0.70 K, and these values are higher than T_{opt} obtained for recombinant exo-inulinase from *A. niger*. No-recombinant exo-inulinase can be used to inulin hydrolysis industrial process that take place at higher temperature.

The activation energy E_a

The calculated energy values for activation of E_a were in the range of 32.01 ± 7.80 to 43.83 ± 4.87 kJ mol⁻¹ for recombinant exo-inulinases. For the *A. niger* no-recombinant exo-inulinase [26], it was found that values E_a are in the range of 25.20 ± 2.29 to 60.95 ± 8.30 kJ mol⁻¹. The lowest value of E_a the inulin hydrolysis by recombinant exo-inulinase means that enzyme catalysis is easier than for inulin hydrolysis by no-recombinant exo-inulinase.

The activation energy for enzyme deactivation E_d

The values of activation energies of the deactivation reaction of E_d for recombinant exo-inulinases were obtained in the range from 83.92 ± 4.82 to 352.44 ± 14.26 kJ mol⁻¹ (Table 3). The highest deactivation energy E_d value was obtained for the fungal recombinant exoinulinase *Kluyveromyces marxianus*, which together with an endo-inulinase gene from *A. niger* F4 were cloned and expressed in *Y. lipolytica* Po1h [20]. In this case, not only the recombinant exoinulinase was obtained, but also such an effect of synergistic connection between exo-inulinase and endo-inulinase that

Table 4 The statistical data obtained by determining the parameters of recombinant exo-inulinases

Fig.	R^2	SSE	p			F	P	References
			E_d	β	T_{opt}			
1	0.9783	0.1287	< 0.0001	< 0.0001	0.0020	112.57	< 0.0001	[14]
2	0.9834	0.0561	< 0.0001	< 0.0001	0.0029	147.91	< 0.0001	[15]
3	0.9522	0.1570	< 0.0001	< 0.0001	0.0075	79.75	< 0.0001	[16]
4	0.9261	0.1442	< 0.0001	< 0.0001	0.0090	31.33	0.0015	[17]
5	0.9314	0.1404	< 0.0001	< 0.0001	0.0080	54.27	< 0.0001	[18]
6	0.9310	0.2113	< 0.0001	0.0001	0.0053	40.50	0.0003	[19]
7	0.9973	0.0300	< 0.0001	< 0.0001	0.0001	1140.12	< 0.0001	[20]

the obtained enzyme is characterized by optimal parameters, predisposing it to industrial use.

For the Arjomand et al. [18] measurements the E_d value was obtained $149.40 \pm 19.30 \text{ kJ mol}^{-1}$ for recombinant inulinase *A. niger* 512 Δ 6SL and this value is 40% lower than that the E_d value obtained for no-recombinant exo-inulinase from *A. niger* in previous work [26] as equal to $245.62 \pm 26.18 \text{ kJ mol}^{-1}$. Mazutti et al. [21] studied directly deactivation of inulinase and have found value $343.9 \text{ kJ mol}^{-1}$ for inulinase *K. marxianus* NRRL Y-7571. The activation energy of the deactivation reaction of E_d the commercial exo- and endo-inulinase of *A. niger* for marked k_d Ricca et al. [22] calculated E_d as equal to $313.47 \text{ kJ mol}^{-1}$. The E_d values are in the range from $80.86 \pm 3.59 \text{ kJ mol}^{-1}$ to $268.66 \pm 24.06 \text{ kJ mol}^{-1}$ for the *A. niger* no-recombinant exo-inulinase [26] and obtained E_d values (Table 3) are within the range of values reported to obtained for no-recombinant exo-inulinase from *A. niger*.

In most of the analyzed cases, recombinant exo-inulinases have lower value of E_d , so they are less thermally stable than that no-recombinant exo-inulinases from *A. niger* [26].

Knowing the E_d/E_a relationship for a given recombinant exo-inulinase one can choose the enzyme with the highest thermal stability. The higher the E_d/E_a value of a given recombinant exo-inulinase, the more stable the enzyme is.

Conclusions

The method of determining following kinetic parameters: the activation energies E_a and the deactivation energies E_d as well as and the optimum temperatures T_{opt} of inulin hydrolysis reaction by recombinant exo-inulinases from fungi *Aspergillus niger* and *Aspergillus awamori* and from yeast *Kluyveromyces marxianus* and *Kluyveromyces cicerisporus*; based on curves of changes in recombinant exo-inulinases depending on the temperature of hydrolysis was applied.

For the optimum temperatures T_{opt} , the difference between the obtained values is about 10 K. The differences in the calculated values of activation energy of the reaction E_d is equal to about 270 kJ mol^{-1} , the activation energy of the deactivation reaction E_a is equal to about 8 kJ mol^{-1} . Advances in genetic modification research and bioprocess technology have improved inulinase activity, optimal pH and temperature.

The large differences between the energy value E_d for recombinant exo-inulinases result from a different origin of yeast and bacteria strains. Knowledge of presented parameters for recombinant exo-inulinase yeast will allow design, modeling and optimization process of inulin hydrolysis using this enzyme.

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