Thermostability improvement of sucrose isomerase PalI NX-5: a comprehensive strategy

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Abstract

Objective: To increase the thermal stability of sucrose isomerase from *Erwinia rhapontici* NX-5, we designed a comprehensive strategy that combines different thermostabilizing elements.

Results: We identified 19 high B value amino acid residues for site-directed mutagenesis. An *in silico*evaluation of the influence of post-translational modifications on the thermostability was also carried out. The sucrose isomerase variants were expressed in *Pichia pastoris* X33. Thus, for the first time, we report the expression and characterization of glycosylated sucrose isomerases. The designed mutants K174Q, L202E and K174Q/L202E, showed an increase in their optimal temperature of 5 °C, while their half-lives increased 2.21, 1.73 and 2.89 times, respectively. The mutants showed an increase in activity of 20.3% up to 25.3%. The Km values for the K174Q, L202E, and K174Q/L202E mutants decreased by 5.1%, 7.9%, and 9.4%, respectively; furthermore, the catalytic efficiency increased by up to 16%.

Conclusions: With the comprehensive strategy followed, we successfully obtain engineered mutants more suitable for industrial applications than their counterparts: native (this research) and wild-type from *E. rhapontici* NX-5, without compromising the catalytic activity of the molecule.

Introduction

A diet rich in sugars is closely related to the appearance of insulin resistance (type II diabetes) (Chicco et al., 2003; Pagliassotti et al., 2002; Wylie-Rosett et al., 2004). An alternative to sucrose is one of its isomers, isomaltulose. This molecule has the same total caloric value as sucrose but is slowly digested. This feature leads to a low glycemic response and guarantees a prolonged supply of glucose. For this reason, isomaltulose has become a promising nutrient for diabetics and athletes (de Groot et al., 2020; Rubio-Arraez et al., 2017; Sokołowska et al., 2022).

Isomaltulose exists in extremely small quantities in nature and is difficult to synthesize chemically (Kawaguti, 2007). As a consequence, the enzyme sucrose isomerase (EC 5.4.99.11) has been identified as a commercially attractive way for isomaltulose production (Lee et al., 2011). In addition to isomaltulose, the catalytic action of sucrose isomerases derives and cleaves the sucrose molecule into trehalulose, glucose, and fructose (Huang et al., 1998; Véronèse et al., 1999). In this process, the ratio of products will depend on the bacterial strain producing the enzyme and the reaction conditions (Aroonnual et al., 2007; Cha et al., 2009; Duan et al., 2016; Ravaud et al., 2009; Salvucci et al., 2003; Wu et al., 2005).

Among the microbial enzymes capable of producing isomaltulose that have been purified and characterized so far are the native isoforms of *Erwinia rhapontici* NX-5 (Ren et al., 2011), *Erwinia sp*. D12 (Kawaguti et al., 2010), *Serratia plymuthica* ATCC15928 (Véronèse et al., 1999), as well as the recombinant forms of *E. rhapontici* DSM 4484 (Börnke et al., 2001), E. *rhapontici* NX-5 (Li et al., 2011), *Enterobacter sp*. FMB-1 (Cha et al., 2009), *Klebsiella planticola* UQ14S (Wu et al., 2005), *Erwinia rhapontici* WAC2928 (Wu et al., 2005), and *Pantoea dispersa* UQ68J (Li et al., 2011). Despite the variety

of sucrose isomerases that have been identified, all their isoforms are mesophilic enzymes. The low activity and stability of sucrose isomerases at temperatures above 40°C is the bottleneck for its industrial application (Mu et al., 2014).

Biotechnological research to increase the thermostability of enzymes has become an important field in industrial applications (Fágáin, 1995). Numerous strategies have been developed to enhance the thermostability of proteins (Eijsink et al., 2005; Pack et al., 2004). Nevertheless, there is no universal procedure to achieve protein thermostabilization. Despite this, the success of the thermostabilization process is usually associated with increasing the rigidity of the protein structure (Xu et al., 2020). Some factors that can increase rigidity are: the appearance of new hydrogen interactions (Gong et al., 2018; Han et al., 2019; You et al., 2019), disulfide bridges (Bashirova et al., 2019; He et al., 2019; Teng et al., 2019), salt bridges (Vicente et al., 2020; Wang et al., 2020), shortening loops (Qiao et al., 2020), electrostatic surfaces optimization (Dotsenko et al., 2020), greater solvation in specific regions of the protein, better packaging of the molecule (Zheng et al., 2019) and glycosylation (Han et al., 2020; He et al., 2019).

A method designed for identifying hot spots, to strength rigidity is the B factor methodology (Han et al., 2019; He et al., 2019; Parthasarathy et al., 2000). The B factor profile of a protein represents the diffusion of atomic electron densities around its equilibrium positions due to thermal movement and positional disorder (Yuan et al., 2003). As a result, the B values provide information on the flexibility of amino acid residues and protein stability (Reetz et al., 2006). However, B factor profile analysis requires crystallographic information of the target protein. However, this might seem a limitation; today, more than 700,000 proteins have a reported crystal structure in NCBI. In Protein Data Bank, there are 25 structures of sucrose isomerase isoforms with or without interacted chemicals, including the enzymes from *E. rhapontici* NX-5 (Xu et al., 2013).

In this study, a comprehensive strategy focused on combining different principles that contribute to thermostability was followed. The residues to be mutated in the sucrose isomerase PalI NX-5 were identified based on B factors analysis, the prediction of hydrogen bond formation, and molecular docking at the active site. In addition, the potential presence of post-translational modifications influencing target protein thermostability was evaluated *in silico*. Based on this, engineered variants of the sucrose isomerase PalI NX-5 were expressed in *Pichia pastoris* X33, being this research the first report of expression and characterization of glycosylated sucrose isomerases. The engineered mutants showed enhanced thermostability and catalysis.

Materials And Methods

Design and construction of mutants

The sucrose isomerase PalI NX-5 structure was modeled using the Swiss-Model software (https://swissmodel.expasy.org/) and the 4hox crystal structure (Xu et al., 2013). Using the B-FITTER software, the B values of the 600 amino acid residues that make up the enzyme were obtained, from

which the average B value of the molecule was calculated. Then, the amino acid residues whose B factor was between 2.5 and 3.5 times the mean of the protein value were selected. The selection of more rigid amino acids was carried out using the ROSETTA DESIGN server (http://rosettadesign.med.unc.edu). Each selected residue was replaced by the remaining 19 biological amino acids. Using PyMOL software, the appearance of potential interactions, involving mutant residues or their neighborhood, was analyzed. The AutoDockTools-1.5.6 and AutoDock Vina softwares were used to verify the conservation of the enzyme-substrate interaction, and thus the potential affinity of the mutant molecules towards the substrate. Default docking parameters were used, and point charges were initially assigned according to the AutoDock semi-empirical force field. The mutants with the most promising (*in silico*) properties were obtained by site-directed mutagenesis using as a starting point the native protein. The possible presence of post-translational modifications influencing target protein thermostability was evaluated using Prosite (https://prosite.expasy.org/) (De Castro et al., 2006).

Strains, vectors, and materials

To eliminate unwanted restriction sites, the native and mutant sequences of the *pall* NX-5 gene were first subjected to silent point mutations. These genes were synthesized and cloned in the yeast expression vector pPICZα A (Invitrogen®), generating the plasmids pSINX5n, pSINX5-L202E, pSINX5-K174Q, and pSINX5-L202E-K174Q. *P. pastoris* X33 strains (provided by CIAD, Cd. Cuauhtémoc, Chih. Mexico) were used for the recombinant expression of native and mutant PalI NX-5. *Escherichia coli* DH5α was used for the plasmids propagation.

Culture conditions and purification of sucrose isomerase variants

E. coli DH5α was cultured at 37°C in Luria–Bertani (LB) broth (10 mg tryptone l^{-1}, 5 mg yeast extract l^{-1}, 10 mg NaCl l^{-1}) and LB plates (15 mg agar l^{-1}) supplemented with zeocin (25 μg·ml^{-1}) as the selective marker. *P. pastoris* X33 was cultured at 30°C and 250 rpm on YPD (1% yeast extract, 2% peptone, 2% dextrose) broth and YPD plates (20 mg agar ml^{-1}) supplemented with zeocin (200 μg·ml^{-1}) as the selective marker. Induction of expression of sucrose isomerase PalI NX-5 variants was performed as proposed by Jiang et al. (2008) using BMMY media (PBS 100 mmol·l^{-1} pH 6.5, 1% yeast extract (Sigma-Aldrich®), 2% peptone (Sigma-Aldrich®), 1.34% YNB (Sigma-Aldrich®), 400 μg biotin l^{-1} (Sigma-Aldrich®) and 0.5% methanol (Sigma-Aldrich®)).

Solid ammonium sulfate was added to the yeast culture supernatants to 60% saturation. The samples were incubated with shaking (100 rpm) for 2 h at 4°C. The active precipitate was collected by centrifugation at 10,000×g for 30 min and dissolved in 1X PBS (pH 7.4). The samples were dialyzed against 1X PBS pH 7.4 for 24 h. After dialysis, protein extracts were purified using a high-affinity Ni-Charged Resin (GenScript®) according to the manufacturer's instructions to obtain purified sucrose isomerase PalI NX-5 variants.

SDS-PAGE and protein assay

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% (w/v) acrylamide gel as described by Laemmli (1970). Protein bands were visualized by Coomassie brilliant blue R-250 staining. For carbohydrate staining, the Glycoprotein Detection Kit (Sigma-Aldrich®) was used. The molecular weight marker PageRuler™ Plus (ThermoFisher) (10–250 kDa) was used to estimate the molecular weight of the sucrose isomerase PalI NX-5 variants. Protein concentration was determined by the Lowry method with bovine serum albumin (Sigma-Aldrich®) as the standard.

Characterization of sucrose isomerase PalI NX-5 variants

Sucrose isomerase activity assay

The activity of sucrose isomerase was measured at 30°C in 50 mM citric acid/sodium phosphate buffer (pH 6.0) containing sucrose at a final concentration of 292 mM. Specifically, 100 µl diluted enzyme solution was mixed with 400 µl of an assay solution and incubated for 15 min. The reaction was stopped by boiling the mixture in a 100°C water bath for 5 minutes (Zhang et al., 2002). Afterward, the sucrose, isomaltulose, trehalulose, glucose, and fructose contents of the assay mixture were quantified using a HPLC system equipped with a refractive index detector, as described below. One unit of sucrose isomerase activity was defined as the amount of enzyme that produced 1 µmol of isomaltulose or trehalulose per min under the conditions described above.

Effects of pH and temperature on enzyme activity

To determine the optimal pH for enzyme activity, assays were carried out in 50 mM buffers containing citric acid/sodium phosphate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), or glycine/NaOH (pH 8.0 to 9.0). The optimal temperature was determined between 15°C and 70°C in 50 mM citric acid/sodium phosphate buffer (pH 6.0), using 292 mM sucrose as the substrate.

To test enzyme stability, the purified enzyme was incubated at 40°C. Aliquots were removed at different incubation times, and the residual activity was measured at 30°C in the same buffer using 292 mM sucrose as substrate. The initial activity before incubation was taken as 100%. All enzymatic assays were performed in triplicate, and the results are expressed as the mean ± standard deviation.

Measurement of Kinetic Parameters

To determine the kinetic parameters, sucrose isomerase was measured by incubating the purified enzyme with different sucrose concentrations (110, 120, 135, 146, 190, 234, 292, 438, 584, 730, 876 y 1010 mM) under standard assay conditions. The Km and Vmax values were determined by Michaelis–Menten plots. All kinetic parameters presented in this study were determined in triplicate, and the results are expressed as the mean ± standard deviation.

HPLC analysis

The analysis of the sucrose, isomaltulose, trehalulose and glucose contents of the reaction mixture was carried out with an Agilent 1100 high-performance liquid chromatography (HPLC) system.

Chromatographic separation was achieved at a flow rate of 0.8 ml min^{-1}, temperature: 25°C, mobile phase: acetonitrile/water (71:29) using the Zorbax NH2 analytical 4.6 x 250 mm 5µm Agilent® column. A refractive index detector was used.

Results And Discussion

Design of sucrose isomerase PalI NX-5 mutants

Identification of potential residues to be substituted

There is no universal procedure for the thermostabilization of an enzyme. However, in this research, we follow a comprehensive strategy that combines different principles contributing to thermostability. For this, the B factor profile of the crystalline structures 4hox, 4how and 4hoz (Xu et al., 2013) of the sucrose isomerase PalI NX-5 was analyzed. Thus, 19 amino acid residues with B values between 2.5 and 3.5 times the average of the molecule (average B factor: 16.5) were identified (Supplementary Table 1). Using the ROSETTA DESIGN software, these residues were subjected to individual substitutions. It has been observed that the prevalence of hydrogen bond networks in mesophilic enzymes is lower than in thermophilic ones (Tompa et al., 2016). In addition, the hydrogen bond is the most referenced physicochemical interaction to explain the increase in thermostability (Querol et al., 1996). For this reason, the appearance of potential interactions involving the mutant residues, or their neighborhood, was analyzed *in silico*. Only in the substitutions Leu202Glu, Lys174Gln, and Asp195Glu new interactions or reinforcement of the existing ones were observed.

As shown in Fig. 1a, when Glu occupied position 202, a new interaction between Glu202 and Lys248 is predicted. A restructuring of the hydrogen bond network is also observed in the vicinity of Asp211, which now interacts with Ala190 instead of Tyr204. The readjustment observed in the hydrogen bonds network could imply an increase in the thermal stability of the molecule since, in addition to the appearance of a new interaction (Glu202–Lys248), the network now encompasses more distant residues belonging to elements different from the side-chain. When comparing the neighborhood of the Lys174 and Gln174 residues (Fig. 1b), no changes were observed in the interaction distances with the His177 residue. However, a new interaction between Asp175 and Gln174 was predicted. For the Asp195Glu substitution (Fig. 1c), a repositioning of the Asp194-Ser197 hydrogen bond was observed, settling between Asp 194 and Lys 196. Associated with this restructuring, a reduction in the interaction distance was predicted, going from 2.5 Å to 2.1 Å. Precisely, Zheng et al. (2020) reported that for the occurrence of hydrogen bonds, the distance between the proton acceptor and the hydrogen atom must be less than the sum of the Van der Waals atomic radii of the two atoms in question; in the case of the oxygen-hydrogen combination, this distance is 2.5 Å (Pauling, 1960). Thus, the reduction in the distance of the hydrogen bond would imply a strengthening of this interaction.

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Two elements that are interesting up to this point are: first that the substitutions for which interactions were observed do not correspond to the residues that presented the highest values of B factor, such as Asp175 and His177, confirming that the thermal stability is achieved through cooperative optimization of several factors rather than predominant interaction. Second, of the three substitutions where new interactions were observed in the neighborhood, one corresponds to Gln, a residue on which the protein statistics of Khan et al. (2019) indicate a poor prevalence in thermophilic molecules, while Warren et al. (1995) reported that the presence of Gln in helices of thermostable proteins is greater than in mesophilic proteins. Given this ambiguity, the practical evaluation will be conclusive.

The conservation of the enzyme-substrate interaction was verified *in silico* in the mutants Leu202Glu, Lys174Gln, Asp195Glu, and their combinations. In molecular docking analysis at the catalytic site, glucose was used instead of sucrose. It was taken into account that Véronèse et al. (1999) showed that glucose is a competitive inhibitor of sucrose; and that Véronèse et al. (1998) previously deduced the glycosyl binding of sucrose to the active site, assuming that free glucose binds to the enzyme at the same site and in the same way. The highest affinities (Viewed as |Energy < 0|) enzyme-glucose were predicted for the L202E, L202E/K174Q, K174Q/D195E, and L202E/D195E variants (Table 1). These variants presented interaction energies that were 6% higher than the native enzyme; the highest increase observed among the 1440 configurations analyzed. Based on the *in silico* data shown in Table 1, the mutants selected for recombinant expression and subsequent evaluation were the L202E, L202E/K174Q, and K174Q variants. Figure 2 shows the predicted interactions between D-glucose and the three variants L202E, L202E/K174Q, and K174Q, including the native enzyme. We were able to identify interactions between glucose and residues R325, D241, E295, D369, and H368. Zhang et al. (2003) have previously reported the catalytic function of these residues. These results suggest the potential conservation of the enzyme-substrate interaction.

Table 1
Interaction energies at the catalytic site of sucrose isomerase PalI NX-5 variants and the D-glucose molecule. Default docking parameters were used, and point charges were initially assigned according to the AutoDock semi-empirical force field.

Variant	Affinity (kcal/mol)	Condition
Native	-6.3	Seed: 11, state: 1
L202E	-6.7	Seed: 1, state: 1
K174Q	-6.5	Seed: 7, state: 1
D195E	-6.3	Seed: 11, state: 2
L202E/K174Q	-6.7	Seed: 17, state: 1
K174Q/D195E	-6.7	Seed: 10, state: 1
L202E/D195E	-6.7	Seed: 10, state: 1
K174Q/L202E/D195E	-6.3	Seed: 1, state: 1

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Potential N-glycosylation of sucrose isomerase variants

Through Prosite (https://prosite.expasy.org/) (De Castro et al., 2006), we evaluated the potential presence of post-translational modifications that influence thermostability. Three potential sites to be N-glycosylated were identified in the sucrose isomerase PalI NX-5 variants. The first of these sites is found at position 144NHT146, including the catalytic residue H145, just inside the catalytic pocket. Taking into account that in the "endoplasmic reticulum – Golgi apparatus" pathway, the previously assembled glycans are added to the growing polypeptide chain (Colley et al., 2015), it would be expected that the glycosylation of residue N144 leads to obtaining of a glycoform with a non-functional tertiary structure. However, if the recombinant expression of sucrose isomerase PalI NX-5 were mediated by the α factor secretion signal from *Saccharomyces cerevisiae*, the translocation to the endoplasmic reticulum would be post-translational (Barrero et al., 2018), so the protein it would fold in the cytosol, and residue N144 would not be available to be glycosylated. The remaining two potential N-glycosylation sites (530NNS532 and 573NNS575) are located on the surface of the C-terminal domain of the molecule. Fortuitously, the position of these consensus sequences for glycosylation is very close to hot spots of high B value. Specifically, the potential site 530NNS532 is located in ten residues from the 541GAE543 hot spot, while the site 573NNS575 is within the high B factor segment from residue E553 to residue R587.

Reports such as those by Colley et al. (2015), Katla et al. (2019), Benoit et al. (2006) and (Zou et al., 2013) agree that the presence of glycosylation in a protein constitutes, in a general sense, a contribution to the stability of the molecule, whether thermal, against pH, presence of proteases or stress physiological.

Specifically, Benoit et al. (2006) isolated a feruloyl esterase A from *Aspergillus niger* (naturally glycosylated), which is more thermostable than its non-glycosylated counterpart expressed in *E. coli*. On the other hand, Zou et al. (2013) observed that the structural and functional stability of β-glucuronidase from *Penicillium purpurogenum* was improved after its glycosylated expression in *P. pastoris* GS115 compared to its wild-type counterpart. However, as evidenced in the works of Han et al. (2020) y Hu et al. (2019), the effect of this post-translational modification is very complex and not always predictable or beneficial.

It has been observed that the stabilization caused by glycosylation is closely associated with the entropy and with the positions of the glycosylation sites in the protein (Helenius et al., 2004; Shental-Bechor et al., 2008). Glycans bound to flexible regions, in general, would confine the conformational space and stimulate protein entropy reduction, thus improving conformational stability at high temperatures (Adney et al., 2009; Dotsenko et al., 2016). Taking this context into account, it is possible to hypothesize that, since residues N530 and N573 are found in loop segments with high B factor, the addition of glycans to these positions will have a positive effect on stability compared to what we would expect for the wild-type variant of *E. rhapontici* NX-5 (not glycosylated), and even complement the substitutions made at positions K174Q and L202E.

Site-directed mutagenesis and expression vectors

We started from the amino acid sequences of the four variants of sucrose isomerase PalI NX-5 (native, L202E, K174Q, and L202E/K174Q) previously analyzed. Using the Sequence Manipulation Suite software (Stothard, 2000), the reverse translation of each of these sequences was carried out, for which the usage codons of *P. pastoris* were used. The variants of the *pall* NX-5 gene were inserted into the genomic integration vector pPICZα A (INVITROGEN®), thus obtaining the constructions pSINX5n, pSINX5-L202E, pSINX5-K174Q and pSINX5-L202E-K174Q (Supplementary Fig. 1). These constructions were used for the transformation of *P. pastoris* X33 and consequently enabling the glycosylation of the variants of the sucrose isomerase PalI NX-5.

Expression analysis of sucrose isomerase PalI NX-5 variants

P. pastoris X33 colonies transformed with the pSINX5n, pSINX5-L202E, pSINX5-K174Q, and pSINX5-L202E-K174Q constructs were cultured in a protein induction medium (see “Materials and methods”), the supernatant was collected, dialyzed and purified using a high-affinity Ni-Charged Resin (GenScript®). Aliquots of purified sucrose isomerase PalI NX-5 variants were analyzed by SDS-PAGE. After Coomassie blue staining, the presence of bands at approximately 70 kDa was confirmed (Fig. 3a, lanes 3, 4, 5, and 6), close to the molecular weight predicted by Protein Calculator v3.4 (http://protcalc.sourceforge.net/). Interestingly, at least two bands were observed in the purified sucrose isomerase PalI NX-5 (Fig. 3a, lanes 3, 4, 5, and 6). One hypothesis for this behavior could be the capacity of *P. pastoris* to glycosylate secreted proteins. Thus, the bands observed for each of the purified PalI NX-5 variants could correspond to different levels of glycosylation reached during cell cultures.

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To qualitatively verify the presence of glycoproteins in the purified samples, the Glycoprotein Detection Kit (Sigma-Aldrich®) was used. In Fig. 3b, the protein bands with glycans attached to their surface can be seen stained in purple. When comparing the migration profiles of the purified ones (Lanes 3, 4, 5, and 6) shown in Fig. 3a and 3b, it is verified that the observed bands correspond to glycoproteins. Thus, at least two main glycoforms of the sucrose isomerase PalI NX-5 variants would be obtained, this being the first report of expression and characterization of glycosylated sucrose isomerases.

Influence of temperature on the activity and stability of recombinant sucrose isomerase PalI NX-5 variants

The influence of temperature on the isomerase activity of PalI NX-5 variants was studied in the range of 15 to 68°C (Fig. 4a). The native variant of PalI NX-5 showed its maximum activity at 30°C, coinciding with its counterparts: wild-type (from *E. rhapontici* NX-5) (Ren et al., 2011) and recombinant (from *E. coli* BL21(DE3)) (Li et al., 2011). Thus, the glycosylated expression of the native variant apparently had no influence on the optimum temperature observed. In this sense, Hua et al. (2014), Han et al. (2014b) and Petrescu et al. (2004) have documented that N-glycosylation of recombinant enzymes expressed in yeast may not have significant effects on activity. Likewise, Han et al. (2014a) reported that some N-glycosylation sites are not essential for folding or cannot affect enzymatic activity due to their distance from the active site of the recombinant enzyme; thus, the presence (or absence) of the glycans has a minimal effect on enzyme activity.

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The K174Q, L202E, and K174Q/L202E mutants showed an increase in the optimal temperature of 5°C (Optimal temperature: 35°C). Furthermore, its relative activity at 40°C was 96% of the observed optimum, while that of the native variant was only 87% of its optimum. Thus, the simple effect of the mutations made on the PalI NX-5 isoform, or its combination with the glycosylation of the molecule, resulted in the improvement of this indicator parameter of thermostability. The improvements achieved in the activity profile versus temperature (Fig. 4a) are not only important in the context of the PalI NX-5 isoform since most sucrose isomerases have a temperature optimum between 30 and 35°C (Mu et al., 2014; Zhan et al., 2020; Zhang et al., 2019; Zhang et al., 2018).

The thermostability of the PalI NX-5 variants was evaluated by incubating the enzymes in 50 mM sodium phosphate/citric acid buffer (pH 6.0), at a temperature of 40°C. Their residual activities were then tested at different incubation times. As shown in Fig. 4b, the thermostabilities of the mutants were higher than that shown by the native enzyme, with the K174Q/L202E variant showing the best performance. At 40°C and pH 6.0, the half-life of the native variant was 10.1 minutes. In comparison, the half-lives of the K174Q, L202E, and K174Q/L202E variants were 22.3 min, 17.5 min, and 29.2 min, respectively, which are 2.21, 1.73, and 2.89 times higher than those of the native variant. Both the increase observed in the optimal temperature of the mutants (Fig. 4a) and half-life times (Fig. 4b) confirm that the comprehensive

strategy outlined in this research to achieve thermostabilization of sucrose isomerase PalI NX-5 was successful.

Two central factors that influence the thermal stability of a protein are the increase of hydrogen bonds (Bi et al., 2020; Masakari et al., 2020; You et al., 2019) and salt bridges (Bian et al., 2015). For this reason, we analyzed the possible appearance of these interactions and observed that associated with the K174Q and L202E substitutions was a reinforcement of the bonds network. Probably, the optimization of the bonds network and its combination with the rigidity provided by N-glycosylation are the keys to the improvement achieved in the thermostability of sucrose isomerase PalI NX-5. Furthermore, with the L202E substitution, we removed an apolar residue, replacing it with a polar/charged one. Khan et al. (2019), when analyzing the relative abundance of amino acids in proteins, observed that charged residues (Charged residues > Glu > Basic residues, abundance) had the highest abundance in thermophilic proteins than in mesophilic ones. In this sense, Chakravorty et al. (2017) state that charged amino acids are involved in the appearance of ionic interactions and the formation of salt bridges, hence their positive contribution to thermostability. Also, Wang et al. (2020) confirmed the role of salt bridges in enhancing the thermal stability of the r27RCL enzyme from *Rhizopus chinensis*; precisely, the appearance of a new salt bridge between residues Glu292 and His171 of the mutant m31 was one of the factors that conditioned the improvement of the parameters indicating thermostability. Likewise, Vicente et al. (2020), after implementing the Ser264Lys substitution, refer to the appearance of a new salt bridge (with the Asp205 residue) as a stabilizing interaction in a laccase variant with high redox potential from white rot fungi.

Influence of pH on the activity of recombinant sucrose isomerase PalI NX-5 variants

The study of the influence of pH on the activity of recombinant sucrose isomerase PalI NX-5 variants was carried out for pH between 4 and 9 (Fig. 4c). The native PalI NX-5 variant showed its maximum activity at pH 6.0, which was consistent with its wild-type counterpart expressed in *E. rhapontici* NX-5 (Ren et al., 2011). In general, the activity profiles vs. pH of native and wild-type PalI NX-5 were found to be very similar. These results indicate that glycosylation of the molecule did not significantly affect the activity profile against pH, which was also observed by Han et al. (2020) during the improvement of the thermal stability of the endoglucanase CTendo45 from *Chaetomium thermophilum*.

The mutants and the native variant showed an optimal pH value of 6.0. In addition, the substitutions made did not substantially affect the activity profile against pH. Analogous behavior was observed by Duan et al. (2016) during the thermostabilization of sucrose isomerase PalI AS9. Similarly, Wang et al. (2020) did not observe changes in the profile of enzymatic activity against pH after making the substitutions S142A, D217V, Q239F, and S250Y, in the lipase r27RCL from *Rhizopus chinensis*. However, Wang et al. (2020) found differences when analyzing the stability of the r27RCL lipase variant against pH. Also Khan et al. (2019), during the thermostabilization of the mesophilic lipase of *Bacillus subtilis*, reported changes in the enzymatic activity profile against pH.

Few research addresses mutagenesis directed at changing the optimal pH of enzymes. In general, the proposed strategies focus on modifying the surface charges of the molecule (Yang et al., 1993), changing the values of the acid dissociation constants of the catalytic residues (Dey et al., 2018; Li et al., 2019), and cavity filling (Nielsen et al., 2000). In particular, Shi et al. (2022) replaced amino acids near catalytic residues to modify the optimal pH of the β-galactosidase from *Aspergillus oryzae*. For this, it was taken into account that in enzymes with acid-base catalysis, such as sucrose isomerases (Mu et al., 2014), the residues of the active center are required to be in a catalytically adequate protonation state. Thus, a possible explanation of the behavior observed in Fig. 4c is the fact that the substitutions made (K174Q and L202E) are far from the catalytic residues (Glu277, proton donor, and Asp223, nucleophile) without affecting their protonation state.

Figure 4c shows that only at pH higher than 8 or lower than 5 considerable activity losses occur. This behavior, together with the observed improvement in thermostability, gives the PalI NX-5 mutants great flexibility in industrial applications. Be it the case in the context of a sugar mill, where operating fluctuations demand flexible biocatalysts, or in mitigating the risk of microbial contamination by adjusting the operating pH of an enzymatic reactor.

Evaluation of the kinetic parameters of the recombinant sucrose isomerase PalI NX-5 variants

The kinetic parameters of the recombinant PalI NX-5 sucrose isomerase variants were measured at 30°C. As shown in Table 2, compared to native PalI NX-5, the Km values of K174Q, L202E, and K174Q/L202E mutants decreased by 5.2%, 7.9%, and 9.4%, respectively. This implies that, concerning the native variant, the mutants present an increase in the affinity for the substrate. The PalI NX-5 variants analyzed in this investigation showed Km values in the same order of magnitude as their counterparts: wild-type (Ren et al., 2011) and recombinant (Li et al., 2011). Furthermore, regarding the report by Ren et al. (2011), the native variant and the mutants K174Q, L202E, and K174Q/L202E increased their specific activity by 14.4%, 25.3%, 20.3%, and 20.4%, respectively. Finally, the mutants showed improved catalytic constants (kcat) and catalytic efficiencies (kcat/Km). Remarkably, the catalytic efficiency of the mutants increased by up to 16%.

Table 2
Evaluation of the kinetic parameters of sucrose isomerase PalI NX-5 variants.

Variant	Specific activity (U/mg)	Km (mM)	k_{cat} (s^{-1})	k_{cat} /Km ($s^{-1} \cdot mM^{-1}$)	References
native PalI NX-5	483.8 ± 6.9	255.1 ± 9.6	564.5 ± 1.0	2.21 ± 0.13	This investigation
PalI NX-5 K174Q	529.9 ± 8.4	241.9 ± 6.8	618.2 ± 0.9	2.55 ± 0.15	
PalI NX-5 L202E	509.1 ± 9.9	234.9 ± 5.1	594.0 ± 0.8	2.52 ± 0.08	
PalI NX-5 K174Q/L202E	509.3 ± 8.7	231.2 ± 7.8	594.3 ± 1.1	2.57 ± 0.21	
wild-type PalI NX-5	423	222	NR	NR	(Ren et al., 2011)
Recombinant PalI NX-5	NR	257	NR	NR	(Li et al., 2011)

PLACE Table 2 **HERE.**

The catalytic performance of the mutants was improved. Ren et al. (2020), Duan et al. (2016), Pang et al. (2020), Teng et al. (2019), and Ban et al. (2020) have shown that enzyme activity can be increased when thermostability is improved. Taking this into account, works such as that of Liu et al. (2021) have focused their attention on increasing enzyme activity using optimization strategies for thermal stability. In particular, Duan et al. (2016) justified the increase in activity of the sucrose isomerase PalI AS9 by stating that the substitutions made (E175N and K576D) were far from the catalytic center and the isomerization region. Thus, the mutations could have caused these regions to be more compact, producing a positive effect on the kinetic parameters. The hypothesis that would be applicable in this research since both the substitutions made in the PalI NX-5 (K174Q and L202E), and the potential sites for N-glycosylation (530NNS532 and 573NNS575) are located on the periphery of the molecule, away from the active site. As can be observed in Table 2 the designed mutants present an optimized catalysis with respect to both the native PalI NX-5 variant and the wild-type expressed in *E. rhapontici* NX-5. These results demonstrate that the outlined comprehensive approach can improve thermostability without sacrificing catalytic activity.

The characteristics of the K174Q, L202E, and K174Q/L202E mutants, together with an adequate immobilization and reaction strategy, would allow the reuse of the biocatalyst and, therefore, the reduction of the costs associated with the operation (Choi et al., 2015; Cicerone et al., 2015; Kazlauskas, 2018), thus influencing the stigma of costs when working with enzymes (Wu et al., 2020). In addition, an increase in the operating temperature would lead to a decrease in the viscosity of the reaction mixture,

higher solubility of sucrose, and an increase in the mass transfer rate without drastically affecting the activity of sucrose isomerase during the production of isomaltulose. Another positive consequence would be reducing the risk of microbial contamination (Singh et al., 2015; Turner et al., 2007), elements that will make the operation of an enzyme reactor more effective and efficient.

Conclusion

In this study, a comprehensive strategy that combines different thermostabilizing elements was designed. Residues mutated in the sucrose isomerase PalI NX-5 were identified based on B factor analysis, hydrogen bond prediction, and molecular docking. In addition, the possible presence of post-translational modifications that influence the thermostability of the target protein was evaluated *in silico*. Based on this, sucrose isomerase from *Erwinia rhapontici* NX-5 was expressed in *Pichia pastoris* X33. This research constitutes the first report on expression and characterization of glycosylated sucrose isomerases. The designed mutants K174Q, L202E, and K174Q/L202E, showed an increase in their optimal temperature of 5°C, while their half-lives increased 2.21, 1.73 and 2.89 times, respectively. The catalytic efficiency of the mutants increased by up to 16%. From the point of view of thermostability, engineered mutants are more suitable options for industrial applications than their counterparts: native (this research), wild-type (from *Erwinia rhapontici* NX-5), and recombinant (from *Escherichia coli* BL21(DE3)). The integrative strategy fallowed was successful and its execution did not compromise the catalytic activity of the molecule. In addition, given the holistic nature of this strategy, it could be used in the thermostabilization of other enzymes, with a positive impact in various industries and processes, such as: bakeries, beverage production, detergents, food, pharmaceuticals, and textiles.

Declarations

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Figures

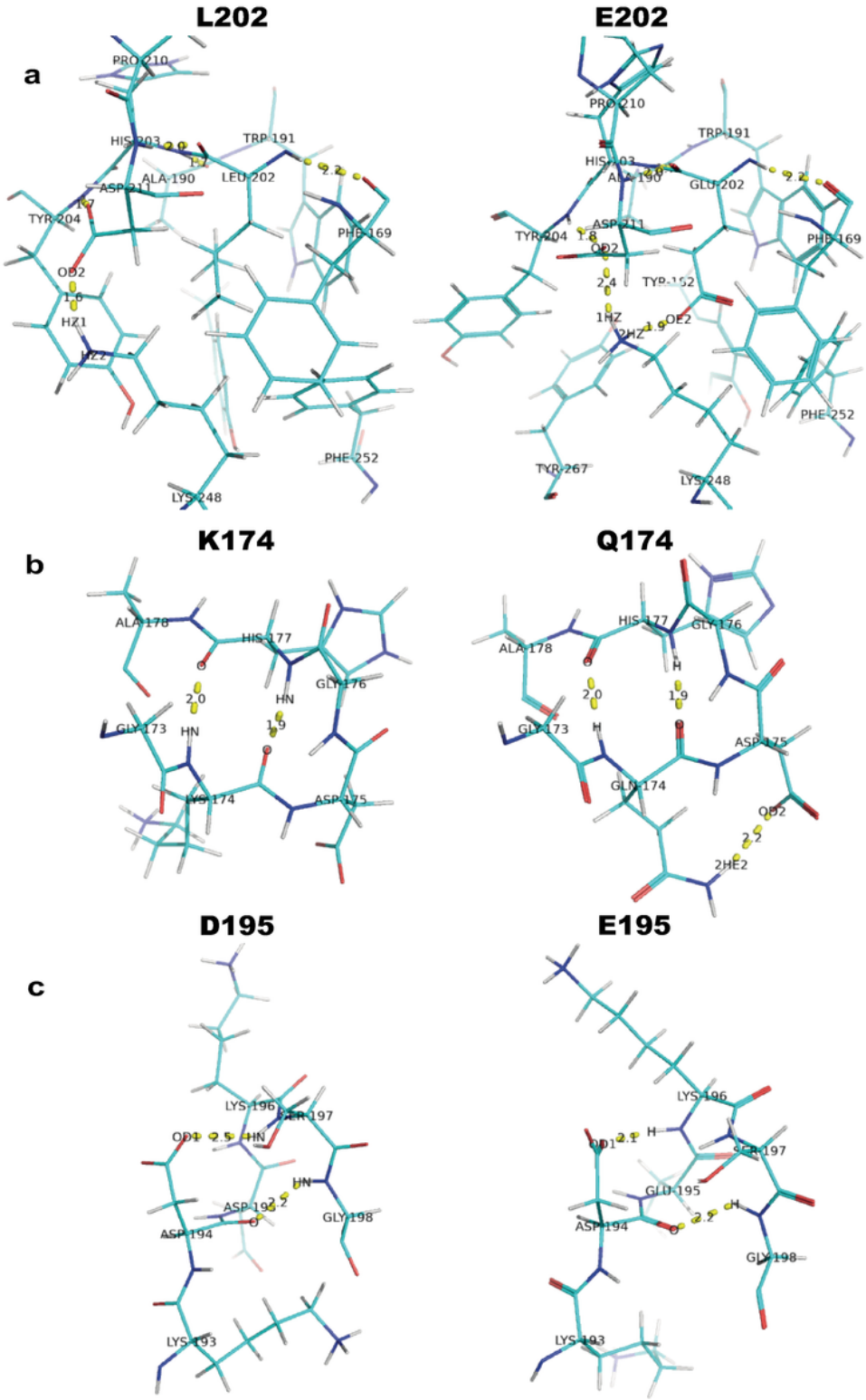

Figure 1

Appearance of potential interactions involving mutant residues or their neighborhood. **a** Residue arrangement and possible interactions in the vicinity of the Leu202 (native) and the Glu202 (mutant). **b** Residue arrangement and possible interactions in the vicinity of the Lys174 (native) and residue Gln174 (mutant). **c** Residue arrangement and possible interactions in the vicinity of the Asp195 (native) and residue Glu195 (mutant).

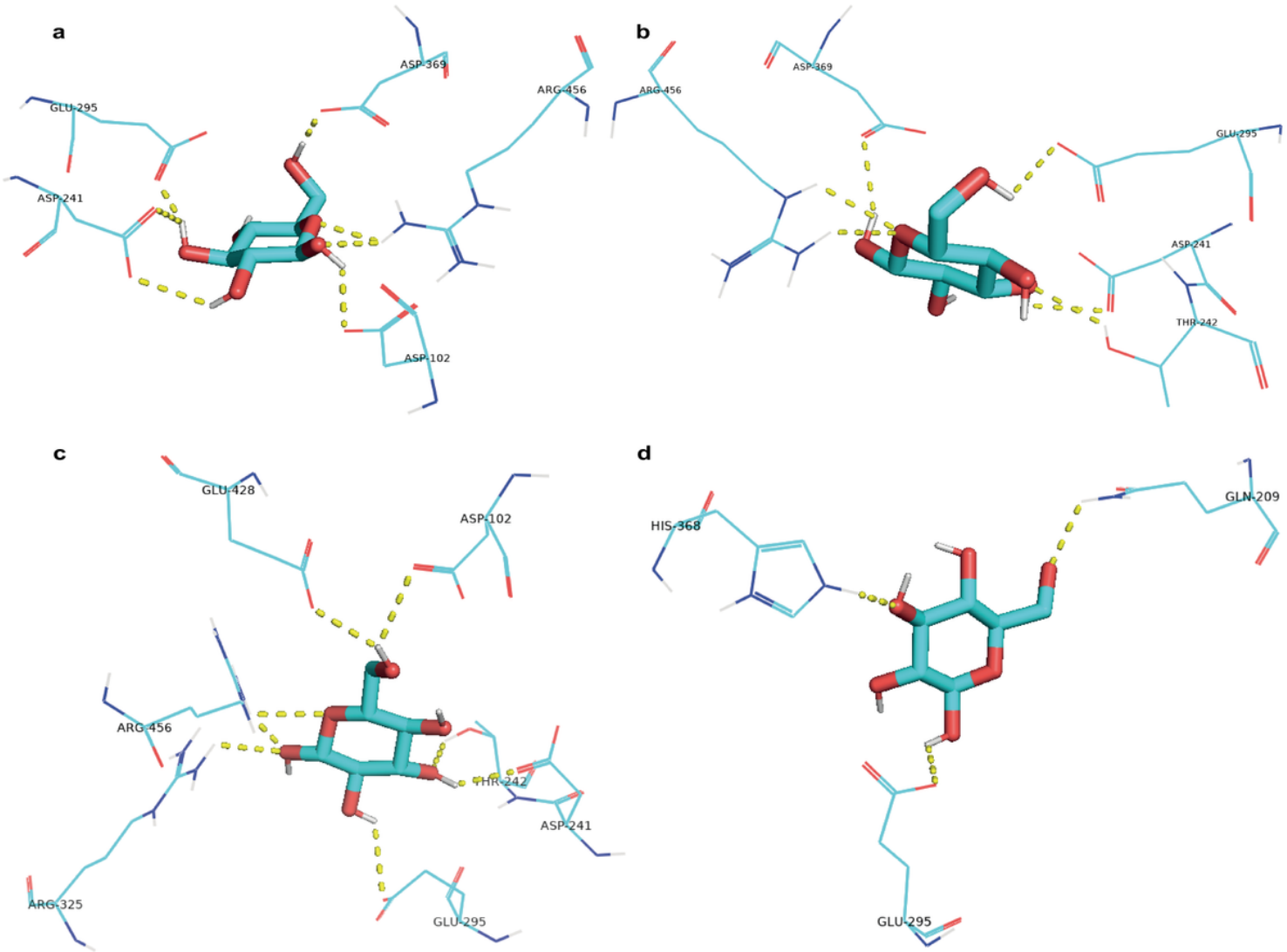

Figure 2

Arrangement of amino acid residues in the active site of PalI NX-5 sucrose isomerase variants and their predicted interactions with the D-glucose molecule (competitive inhibitor of sucrose). **a**Glucose-native enzyme interaction, seed: 11, state: 1, interaction energy -6.3 kcal/mol. **b** Glucose-NX-5 PalI L202E interaction, seed: 1, state: 1, interaction energy -6.7 kcal/mol. **c** Glucose-NX-5 PalI K174Q interaction, seed: 7, state: 1, interaction energy -6.5 kcal/mol. **d** Glucose-NX-5 PalI K174Q/L202E interaction, seed: 17, state: 1, interaction energy -6.7 kcal/mol. We identify interactions between glucose and residues R325, D241, E295, D369 and H368. Zhang et al. (2003) reported the catalytic role of His145, Asp241, Glu295, Asp369 and His368 residues, and the 325RLDRD329 motif in the sucrose isomerase from *Klebsiella sp*. LX3.

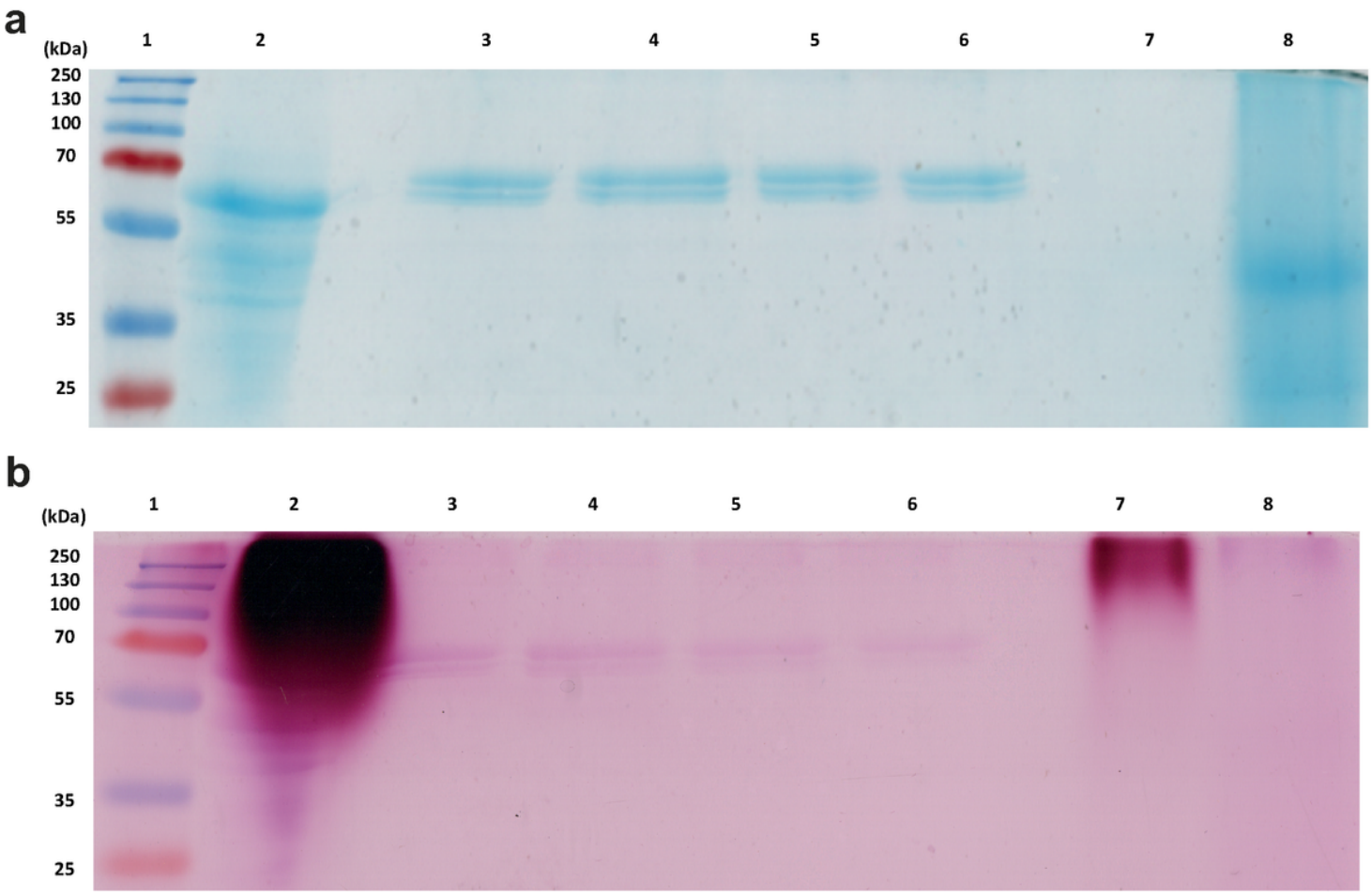

Figure 3

Identification of the variants of the sucrose isomerase PalI NX5 produced in *Pichia pastoris* X33 by SDS-PAGE. **a** Coomassie blue staining of purified proteins, lane 1: PageRuler™Plus molecular weight marker (ThermoFisher), lane 2: commercial laccase from *Trametes versicolor* (Sigma-Aldrich®) (glycosylation positive control), lane 3: sucrose isomerase PalI NX-5 native, lane 4: sucrose isomerase PalI NX-5 K174Q, lane 5: sucrose isomerase PalI NX-5 L202E, lane 6: sucrose isomerase PalI NX-5 K174Q/L202E, lane 7: dialysis of total proteins excreted by non-transformed *Pichia pastoris* X33 (negative control), lane 8: lyticase from *Arthrobacter luteus* (Sigma-Aldrich®) (glycosylation negative control). **b** Glycoprotein staining of purified proteins, lane 1: PageRuler™Plus molecular weight marker (ThermoFisher), lane 2: commercial laccase from *Trametes versicolor* (Sigma-Aldrich®) (glycosylation positive control), lane 3: sucrose isomerase PalI NX-5 native, lane 4: sucrose isomerase PalI NX-5 K174Q, lane 5: sucrose isomerase PalI NX-5 L202E, lane 6: sucrose isomerase PalI NX-5 K174Q/L202E, lane 7: dialysis of total proteins excreted by non-transformed *Pichia pastoris* X33 (negative control), lane 8: lyticase from *Arthrobacter luteus* (Sigma-Aldrich®) (glycosylation negative control).

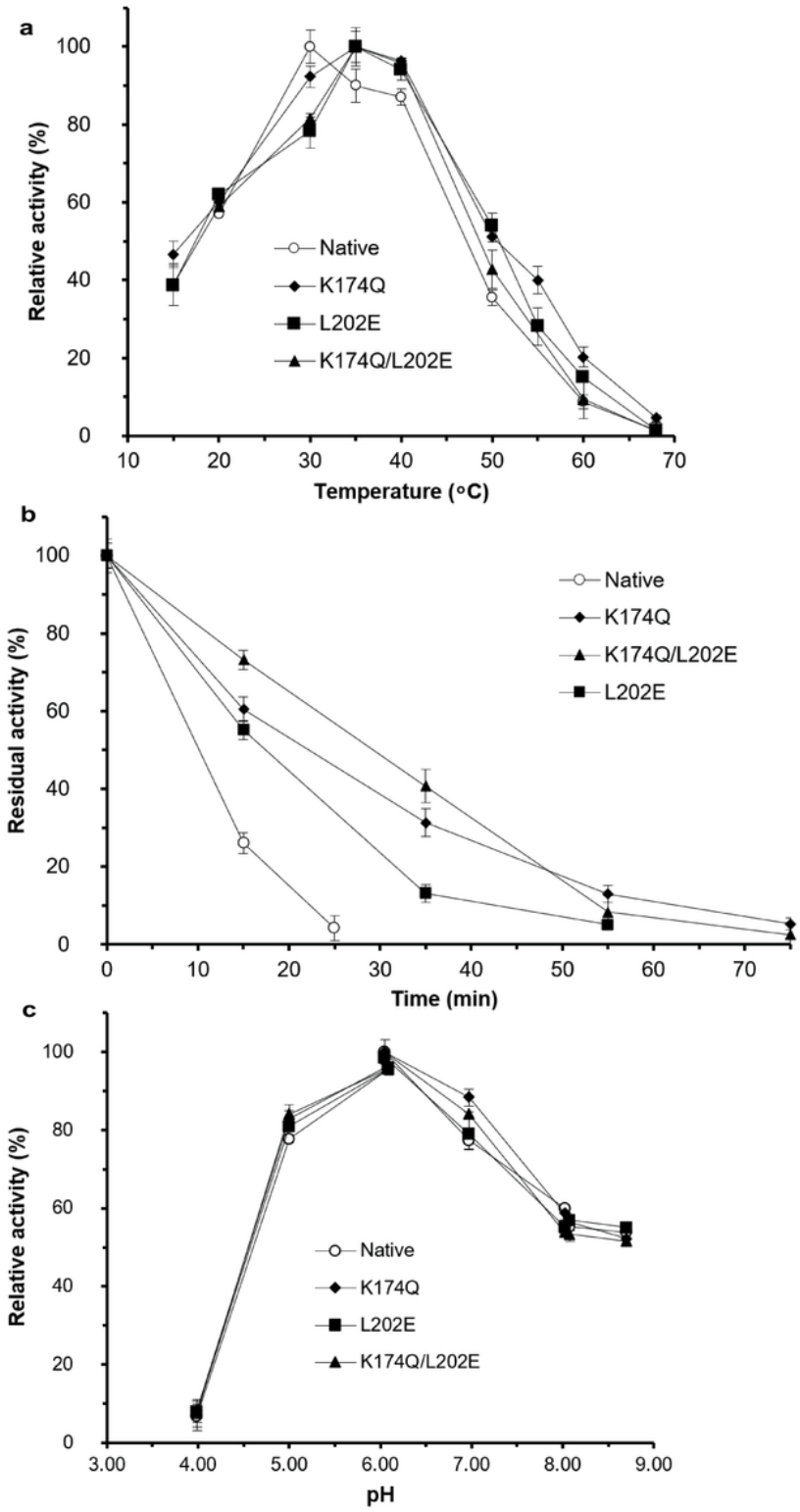

Figure 4

Characterization of sucrose isomerase PalI NX-5 variants. **1a** Influence of temperature on the activity of native and mutant variants of the recombinant sucrose isomerase PalI NX-5. **b** Progression of residual isomerase activity of PalI NX-5 variants incubated at 40 °C. **c** Influence of pH on the activity of native and mutant variants of the recombinant sucrose isomerase PalI NX-5.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.tif
- SupplementaryTable1.docx