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ENHANCEMENT OF THERMAL STABILITY OF *ASPERGILLUS ORYZAE* ALPHA-AMYLASE USING STABILIZING ADDITIVES

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A b s t r a c t. The thermal degradation of *Aspergillus oryzae* α-amylase in the presence of sugars (sucrose, trehalose) and polyols (sorbitol, glycerol) as stabilization additives was investigated. The aim of the study was to test whether it is possible to estimate the heat stability of the enzyme based on the amount of hydroxyl groups provided in a buffer solution from different sources. The thermal inactivation experiments were performed at 68°C. Every additive tested showed a protective effect on the enzyme heat stability, the effect being strongly dependent on the added compound concentration. Among all the stabilizing compounds investigated, sucrose exhibited the greatest protective effect. The decimal reduction time of α-amylase activity increased 33.9 times when 420 mg/ml of sucrose was added to the environment. When the same concentration of trehalose was used, the *D*-value increased 6.4 times compared to the value in the buffer system. The number of hydroxyl groups provided in the enzyme solution could not be related to the D-values for the enzyme thermal inactivation, meaning that the enzyme heat stability was not dependent on the number of hydroxyl (OH) groups.

K e y w o r d s: α-amylase, inactivation, kinetics, stabilization, protective effect

INTRODUCTION

Enzymes as processing aids have been widely used in food industry for more than fifty years. Among them, *Aspergillus oryzae* α-amylase (E.C. 3.2.1.1) is one of the most important. It is used in the brewing industry to increase fermentability of beer worts (including those made from unmalted cereals), in the starch industry to produce high maltose and high DE syrups, in the alcohol industry to reduce fermentation time, in the cereal industry for flour supplementation and improvement of chilled and frozen dough.

The application of enzymes on industrial scale is still limited due to their low stability. As proteins, enzymes are inactivated rapidly by heat and other environmental modifications such as changes in pH, ionic strength. As biocatalysts, enzymes used in industrial food processing have to show an acceptable retention in specific activity during their storage as well as during the process in which they are used. Especially during processes where heat is involved, enzyme stability becomes of utmost importance. The stabilization of enzymes remains one of the most important concerns in modern biotechnology (Aymard *et al*. 2000).

Additional substances such as sugars, polyhydric alcohols, and other organic solvents are used to stabilize enzymes in the liquid form. The addition of sugars and polyols strengthens the hydrophobic interactions among non-polar amino acid residues. These interactions, together with hydrogen bonds and ionic and van der Waals interactions, are essential for maintaining the native, catalytically active structure of the enzyme. Thus, the strengthened hydrophobic interactions make protein macromolecules more rigid, and therefore more resistant to thermal unfolding (Lee *et al*. 1981).

According to Lee *et al*. (1981), the mechanism of enzyme stabilization in the presence of sugars and polyols in aqueous media is an "indirect" action, meaning that the additives do not change the protein conformation but influence the physicochemical properties of the system, such as solvent structure, resulting in protein stabilization. The solvent composition in the immediate domain of the protein is different from that of the bulk solvent. Moreover, the difference is a function of the concentration of co-solvent. As presented by Timasheff *et al*. (1989), co-solvent added to the enzyme aqueous solution is excluded from the protein domain. The free energy of the system increases, which is unfavourable from the thermodynamic point of view. If the protein is unfolded, the surface of the zone of exclusion and free energy increases, which induces less favourable thermodynamic conditions. As a result, the equilibrium of the reaction $N \leftrightarrow U$ (where N represents the native, U reversibly unfolded enzyme) is shifted to the left, toward the native state of the protein (Timashef 1993).

The mechanism of exclusion is different for different compounds, as is the interaction with the unfolded state of the protein. According to Lin *et al*. (1996), in the case of sugars there is a good correlation between the negative preferential interactions and the positive surface tension increment, leading to the suggestion that the stabilization of proteins is due to increase of the surface tension. Glycerol (and other polyols) slightly decreases surface tension of water, and exclusion results from the solvophobic effect. Polyols fit well in the water lattice and their structure permits them to form the proper hydrogen bonds that reinforce water interactions. This makes contacts between non-polar regions of the protein and

the glycerol-water mixture even more entropically unfavourable than contact with water. As a consequence, polyol molecules migrate away from the protein surface (Timasheff *et al*. 1989, Timasheff 1993).

On the contrary, Combes *et al.* (1992) and Graber *et al*. (1989) conclude that polyols stabilize enzymes against thermal denaturation because of direct interactions with proteins, while water organization is very weakly affected by their presence. Graber *et al*. (1989) found polyols to be competitive inhibitors for α-amylase, and the interactions with the active site of the enzyme to be one of the factors of the enzyme stabilization mechanism. Moreover, the stabilizing effect is connected to the number of hydroxyl groups (*n*OH) contained in the molecule – α-amylase affinity for polyhydric alcohols increases as the *n*OH in the molecule increases. Timasheff *et al*. (1989) suggest that the global effect is a balance between the two types of interactions, with preferential exclusion generally predominating.

The aim of the present work was to investigate the protecting effect of sugars and polyols on *Aspergillus oryzae* α-amylase when the latter is submitted to thermal treatment. This study focused especially on the relation between the number of hydroxyl groups provided by the stabilizing additives per one ml of enzymatic medium and the thermal stability of the enzyme.

MATERIALS AND METHODS

Aspergillus oryzae α-amylase Fungamyl 800L^{RM} produced by Novozymes A/S was purchased from Sigma (Germany) in a liquid form with an activity of 800 FAU/g. 1 FAU (Fungal α-Amylase Unit) is the amount of enzyme which dextrinizes 5.26 g of starch dry basis per hour at 37°C and pH 4.7. Protein concentration of Fungamyl 800L was 183 g/l (determined by bicinchinonic acid method, Sigma, Germany).

Addition of stabilizers

Prior to the thermal inactivation experiments, the enzyme was diluted (1.8 FAU ml^{-1}) in 20 mM Bis-Tris buffer pH 7.0, and in sugars (sucrose, trehalose) or polyol (sorbitol and glycerol) solutions – concentrations as presented in Table 1. Control experiments, without sugars or polyols added, were also performed. In order to compare the stabilizing capabilities of the different additives, the concentrations of the latter were calculated (based on the molar mass of the compound and the Avogadro number) in order to provide the same number of *hydroxyl* groups (*n*OH) per volume unit (ml) of the enzymatic medium. Several concentrations of hydroxyl groups (*n*OH/ml) were investigated, as presented in Table 1.

Table 1. Concentrations of sugar and polyol solutions in Bis-Tris buffer, pH 7.0, and corresponding number of hydroxyl groups (nOH ml^{-1}) provided by the additives

nOH ml^{-1}	Sorbitol mg ml^{-1}	Glycerol % (v/v)	Trehalose mg ml^{-1}	Sucrose mg ml^{-1}
0.198×10^{22}	100	8.1	140	140
0.281×10^{22}			200	200
0.314×10^{22}		13.0		
0.396×10^{22}	200			
0.595×10^{22}	300	24.8	420	420
0.725×10^{22}		30.0		
0.992×10^{22}	500	41.0	705	

Isothermal treatment

Solutions (samples of approximately 80 µl) were enclosed in glass capillary tubes (Hirshman, 1.15 mm i.d., 150 mm length) in order to ensure fast heating and cooling. Isothermal experiments were performed in a water bath at a temperature of 68°C for all concentrations of additives. After preset time intervals, samples were withdrawn and cooled in an ice-water bath to stop heat inactivation. Samples were kept in the ice-water bath till the activity measurement.

Assay of α-amylase activity

The activity of α-amylase was measured spectrophotometrically according to the procedure CNP-G3 of Chema Diagnostica (Jesi, Italy). The procedure is based on progressive hydrolysis of 2-chloro-4-nitrophenyl-α-D-maltotrioside, thus gradually releasing 2-chloro-4-nitrophenol which has the maximum absorption at 405 nm. During measurement the temperature was kept constant at 30°C. The enzyme activity was calculated by linear regression analysis of absorption in a function of reaction time. The residual activity of α-amylase subjected to heat treatment was expressed as the ratio between the activity of a heat-treated sample and of a native sample.

Kinetic data analysis

Isothermal inactivation of α-amylase can be described by a first-order kinetic model (Guiavarc'h *et al.* 2002, Terebiznik *et al.* 1997). The integral effect of an inactivation process at constant temperature, where the inactivation rate constant is independent of time, is given in Eq. 1:

$$\ln(A/A_0) = -kt \quad (1)$$

To describe the thermal inactivation kinetics of first-order reactions the TDT model (thermal death time concept) is also commonly used (Van Loey 1997). The *D*-value is defined as a time required, at a constant temperature, to reduce the initial enzyme activity *(A_0)* by 90%. For first-order reactions, the *D*-value is directly related to the rate constant *k* (Eq. 2).

$$D = 2.303/k \quad (2)$$

The decimal reduction time *D* was estimated from the slope of the regression line of log (A/A_0) vs. time.

$$\log(A/A_0) = (-1/D) \cdot t \quad (3)$$

RESULTS AND DISCUSSION

Heat inactivation kinetics

Isothermal inactivation kinetics of *Aspergillus oryzae* α-amylase in a buffer solution and with addition of stabilizing compounds was accurately described by the first-order inactivation model. The correlation coefficient of linear regression of log(A/A_0) versus time varied between 0.94 and 0.99 (Fig. 1-4).

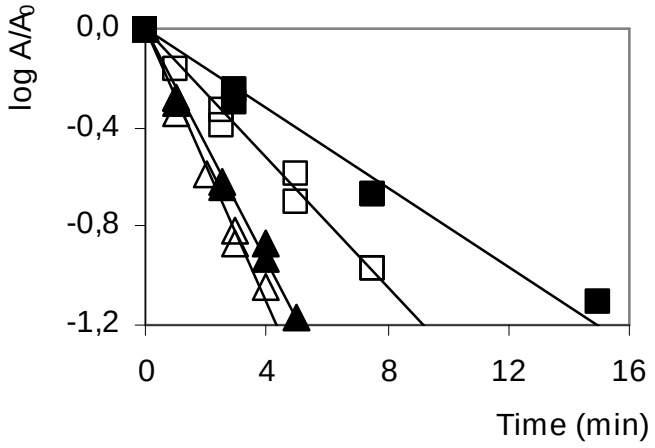

Fig. 1. Isothermal inactivation of α-amylase in 20 mM Bis-Tris buffer, pH 7.0, with addition of sorbitol: 100 mg ml^{-1} (Δ), 200 mg ml^{-1} (▲), 300 mg ml^{-1} (□), 500 mg ml^{-1} (■)

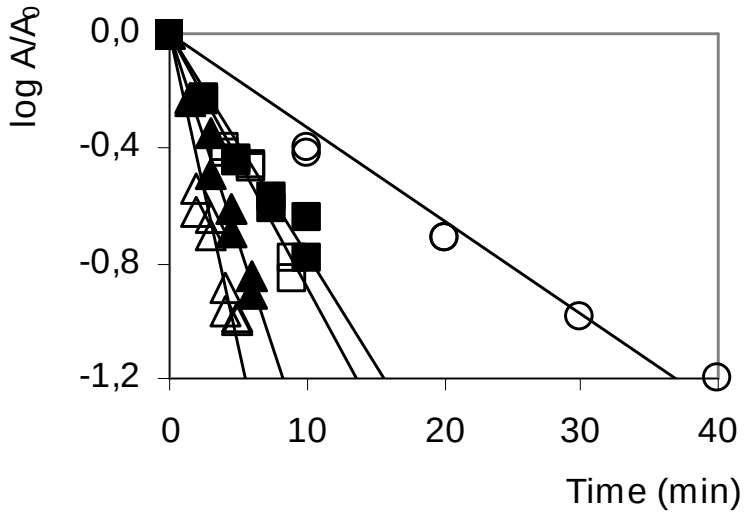

Fig. 2. Isothermal inactivation of α-amylase in 20 mM Bis-Tris buffer, pH 7.0, with addition of glycerol: 8,3 % v/v (Δ), 13 % v/v (▲), 24,8 % v/v (□), 30 % v/v (■), 41 % v/v ()

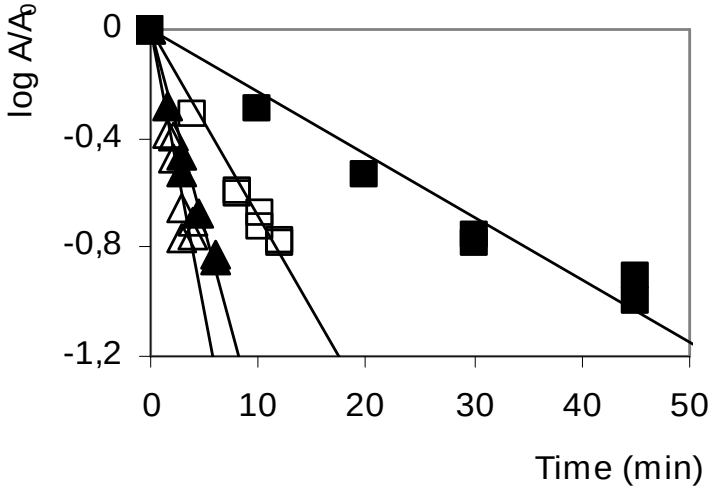

Fig. 3. Isothermal inactivation of α-amylase in 20 mM Bis-Tris buffer, pH 7.0, with addition of trehalose: 140 mg ml^{-1} (Δ), 200 mg ml^{-1} (▲), 420 mg ml^{-1} (□), 705 mg ml^{-1} (■)

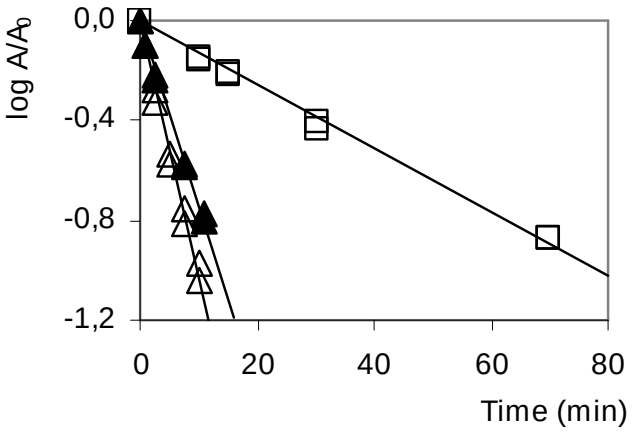

Fig. 4. Isothermal inactivation of α-amylase in 20 mM Bis-Tris buffer, pH 7.0, with addition of sucrose: 140 mg ml^{-1} (Δ), 200 mg ml^{-1} (▲), 420 mg ml^{-1} (□)

Sugars and polyhydric alcohols had a positive effect on the thermal stability of α-amylase, meaning that *D*-value was higher when the additions were applied (Tab. 2).

Table 2. Decimal reduction time (D) for α-amylase isothermal inactivation at 68°C with and without addition of stabilizing compounds, in relation to the number of hydroxyl groups (nOH) provided in the system. (concentrations of sugars/polyols solutions as presented in Table 1)

*n*OH/ml	20 mM Bis-Tris buffer, pH 7.0	Sorbitol	Glycerol	Trehalose	Sucrose
			D (min) at 68°C		
	2.4 (± 0.2)[a]				
0.198×10^{22}		3.8 (± 0.2)	5.3 (± 0.5)	5.4 (± 0.7)	10.2 (± 0.4)
0.281×10^{22}				7.5 (± 0.5)	14.2 (± 0.4)
0.314×10^{22}			6.9 (± 0.4)		
0.396×10^{22}		4.5 (± 0.2)			
0.595×10^{22}		7.9 (± 0.4)	11.4 (± 1.0)	15.4 (± 0.9)	81.3 (± 1.5)
0.725×10^{22}			14.5 (± 1.2)		
0.992×10^{22}		13.2 (± 0.7)	33.7 (± 1.8)	47.4 (± 4.1)	

[a] in brackets – standard error of regression; number of replications – 3.

The most pronounced stabilizing effect was observed with sucrose. When the concentration of 1.2 M (420 mg ml^{-1}) was used, the decimal reduction time *D* increased by a factor of 34 compared to the buffer system without any additives.

Increased thermal stability of lipoxygenase (LOX) in the presence of sucrose was also observed by Busto *et al*. (1999). One to two mol sucrose increased pea LOX stability at 70°C by 400-600%. Polyols like mannitol, sorbitol, erythritol and glycerol were less efficient or had no effect.

The protective effect of all compounds was stronger at higher concentrations (Fig. 5). This behaviour was described before by Graber *et al*. (1989) who observed a protective effect (defined as a ratio of α-amylase half-life with additive to α-amylase half-life without additive) of sorbitol by a factor of 200 in 2.5 M sorbitol solution, and even a factor of 2000 when sorbitol concentration was increased to 4 M. In the current work, the relationship between the concentration of the stabilizing compound and the obtained *D*-value was found to be exponential, with a correlation coefficient R^2 between 0.97 and 0.99 (Fig. 5). Based on the results of the present work, stabilizing compounds can be classified in terms of their protective effect on α-amylase heat stability, as follows: sorbitol < glycerol < trehalose < sucrose, sugars being more favourable than polyols. Each compound showed an exponentially increasing stabilizing effect.

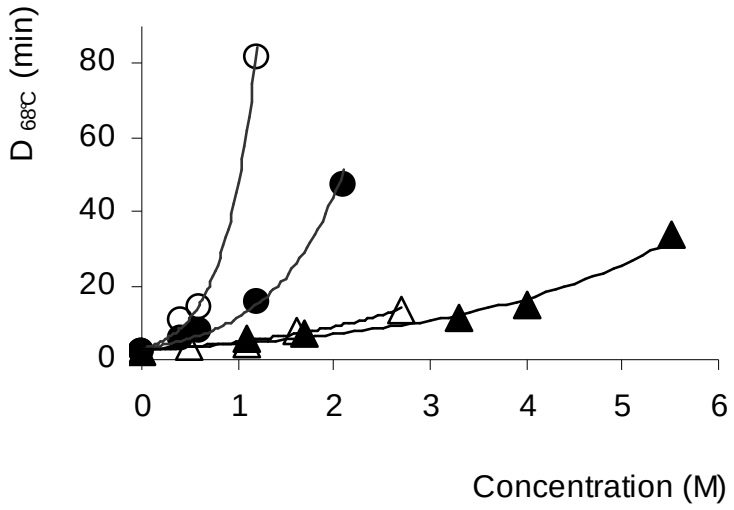

Fig. 5. Decimal reduction time D for α-amylase isothermal inactivation at 68°C in correlation with the concentration of sorbitol (Δ), glycerol (▲), trehalose (●), sucrose (○)

If the mechanism of stabilization is considered to be preferential exclusion of co-solvent from the protein domain, as described before, it can be noticed that sugars, for which the source of exclusion is increase of surface tension, showed a higher stabilizing effect than polyols, for which the source of exclusion is the solvophobic effect. Lin *et al*. (1996) suggested that the increase of surface tension of water in the presence of sugar co-solvent can be responsible for protein stabilization. In the results of current work, sugars, which raise the surface tension of water (Washurn 1928) and do not demonstrate any affinity to or possible interactions with the protein molecules neither in native form nor in an unfolded state, were more effective in the enzyme stabilization. According to Lin *et al*. (1996), due to high correlation between negative preferential interactions and the positive surface tension increment, the stabilization of proteins by the addition of sugars is a result of increased surface tension of water. Polyols have the opposite behaviour and are known to have an affinity to the polar groups of a protein or even are described as competitive inhibitors of α-amylase binding to the active site of the enzyme (Timasheff *et al.* 1989, Timasheff 1993, Graber *et al*. 1989). During protein denaturation, large non-polar regions of the protein become exposed to the solvent. Although the co-solvent is excluded from the protein domain, some molecules can migrate into the zone of preferential hydration and interact with the protein at specific sites (Timasheff 1993).

Several authors have discussed the influence of polyols and sugars, and/or more specifically the influence of hydroxyl groups provided by the latter, on the thermal stability of enzymes (Obon *et al.* 1996, Matsumoto *et al.* 1997, Noel *et al*. 2003). Graber *et al*. (1989) observed that the stabilizing effect of polyols on *Aspergillus oryzae* α-amylase was related to the number of OH groups per molecule (for a given fixed molar concentration of polyol). On the other hand, Guiavarc'h *et al*. (2003) observed, in specific experimental conditions, that the total number of OH groups in the system could be related to the thermal stability of purified pectin methylesterase (PME) independently of the type of additive used. These authors have shown that the *D*-values of purified tomato PME were strongly correlated to the *n*OH provided by polyols and sugars at different concentrations, whereas the source of hydroxyl groups (used in the study) did not influence the thermal stability of PME. They concluded that the thermal stability of purified tomato PME could be predicted based on the type and concentration of additive in the system.

Based on the first observation (number of OH groups per molecule), the stabilizing power of additives used in this study should have been ranked as follows: glycerol (3*OH) < sorbitol, (6*OH) < trehalose, sucrose (8*OH). From Table 2 it can be noticed that it is not the case. The protective effect of stabilizers with the same *n*OH per molecule was different (trehalose, sucrose).

In Table 2 it can be observed that there is no correlation between *D*-value and the number of OH groups per one ml of solution, which is also in disagreement with the observations made by Guiavarc'h *et al*. (2003) discussed above. Different *D*-values are observed for the same *n*OH value achieved with different polyols or sugars, thus showing that the stabilization of *Aspergillus oryzae* α-amylase, as opposed to the stabilization of tomato PME, is sensitive to the source of hydroxyl groups used as a stabilizing substance. A possible reason for such a difference is, as demonstrated by Graber *et al*. (1989), that polyols used during the study act as competitive inhibitors of *Aspergillus oryzae* α-amylase with different inhibition constants, while the same polyols were never demonstrated as being inhibitors of tomato PME. These differences in inhibition constants might generate differences between the stabilizing power of polyols as well as differences between the stabilizing power of polyols and the stabilizing power of sugars (sucrose, trehalose). The later, which are disaccharides, have never been described as being (competitive) inhibitors of *Aspergillus oryzae* α-amylase which only acts on polysaccharides containing three or more 1,4-alpha-linked D-glucose units. These authors showed that interaction of polyols with the active site of *Aspergillus oryzae* α-amylase contributed to its improved thermal stability. It can be noted here that the inhibitory effect of polyols has also been demonstrated on xylanase from *Trichoderma reesei* which, like *Aspergillus oryzae* α-amylase, is also a fungal hydrolase glycosidase (Cobos *et al*. 2003).

CONCLUSIONS

1. The effect of sugars (disaccharides) and polyols on *Aspergillus oryzae* α-amylase thermal stability was investigated.
2. The largest protective effect was obtained with the addition of sucrose. The influence of trehalose, sorbitol and glycerol on the enzyme heat stability was also positive, but less pronounced.
3. The protective effect was strongly related to the concentration of the protective compound, following an exponential function.
4. The number of hydroxyl groups per molecule and the total amount of hydroxyl groups provided by additives to the system (*n*OH) did not correlate with the heat stability of *Aspergillus oryzae* α-amylase.
5. The source of hydroxyl groups was found to be more important, with sugars (especially sucrose) being more effective than polyols for similar *n*OH values.

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ZWIĘKSZENIE STABILNOŚCI CIEPLNEJ α-AMYLAZY Z *ASPERGILLUS ORYZAE* POPRZEZ ZASTOSOWANIE DODATKÓW STABILIZUJĄCYCH

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S t r e s z c z e n i e. Celem pracy było zbadanie wpływu substancji dodatkowych na kinetykę inaktywacji cieplnej α-amylazy z *Aspergillus oryzae.* Badano, czy efekt stabilizujący alkoholi wielowodorotlenowych (sorbitolu i glicerolu) i dwucukrów (trehalozy i sacharozy) związany jest z liczbą grup hydroksylowych występujących w ich cząsteczkach lub z ogólną liczbą tych grup w roztworze. Zastosowane dodatki we wszystkich stężeniach spowodowały wydłużenie czasu dziesięciokrotnej redukcji aktywności enzymu w temperaturze 68°C, co świadczy o ich działaniu stabilizującym na enzym. Zauważalne były różnice w efektywności stabilizacji w zależności od rodzaju substancji oraz jej stężenia. Zastosowane cukrów było bardziej korzystne niż alkoholi wielowodorotlenowych a najbardziej skutecznym dodatkiem stabilizującym okazała się sacharoza. Dodatek 420 $mg \cdot cm^{-3}$ sacharozy pozwolił na uzyskanie ponad trzydziestokrotnego wzrostu czasu dziesięciokrotnej redukcji w porównaniu z roztworem buforowym bez żadnych dodatków. Taka sama ilość trehalozy pozwoliła za sześciokrotne zwiększenie czasu dziesięciokrotnej redukcji. Otrzymane wyniki nie potwierdziły hipotezy o wpływie ilości grup hydroksylowych w cząsteczce substancji dodatkowej lub całkowitej liczbie grup OH w jednostce roztworu na stabilność cieplną α-amylazy. Wykazano, że większy wpływ miało stężenie i rodzaj zastosowanej substancji – w systemach o tej samej zawartości grup OH czas dziesięciokrotnej redukcji różnił się w zależności od zastosowanej substancji.

S ł o w a k l u c z o w e: inaktywacja cieplna, dodatki stabilizujące