

Properties and stabilization of an extracellular α -glucosidase from the extremely thermophilic archaeobacteria *Thermococcus* strain AN1: enzyme activity at 130°C

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Abstract

An extracellular α -glucosidase from the thermophilic archaeobacterium *Thermococcus* strain AN1 was purified 875-fold in five steps (HiLoad Q-Sepharose, phenyl Sepharose, HPHT-hydroxyapatite, gel filtration and Mono Q chromatography) with a yield of 4%. It is a monomer with a molecular mass of about 130 kDa and a pI around 5. At 98°C, the purified enzyme in buffer has a half-life around 35 min, which is increased to around 215 min in presence of 1% (w/v) dithiothreitol and 1% (w/v) BSA. Dithiothreitol (1%, (w/v)) and BSA (0.4%, (w/v)) also substantially increase the enzyme activity. The K_m at 75°C is 0.41mM with pNP- α -D-glucopyranoside as substrate. The substrate preference of the enzyme is pNP- α -D-glucoside > nigerose > panose > palatinose > isomaltose > maltose > turanose, and no activity was found against starch, pullulan, amylose, maltotriose, maltotetraose, isomaltotriose, cellobiose and β -gentiobiose.

A variety of techniques including immobilization (eg. on epoxy and glass beads), chemical modification (cross and co-cross linking) and the use of additives (including polyols, BSA, salts, etc.) were applied to enhance stability at temperatures above 100°C. The half-life could be increased from about 4 min at 110°C to 30 - 60 min at 130°C in presence of 90% (w/v) sorbitol, 1% (w/v) dithiothreitol and 1% (w/v) BSA, and by cocrosslinking with BSA in presence of 90% (w/v) sorbitol. The stabilised enzyme showed good activity at 130°C.

Introduction

Thermostable enzymes are of potential industrial importance and the upper limits of protein stability are of interest biochemically and in terms of the upper temperature limit for life. In this paper we have characterized a thermostable α -glucosidase and attempted to further improve its stability above 100°C.

α -Glucosidases are produced by many thermophilic microbes [1,2] and two types of activity of bacterial glucosidases have been reported: α -D glucosidase (EC 3.2.1.20) and oligo-1,6 α -glucosidase (EC 3.2.1.10) [3].

The α -glucosidase described here is from *Thermococcus* strain AN1, an extremely thermophilic, sulphur reducing archaeobacterium which was isolated from a geothermal spring in Rotorua, New Zealand [4,5].

A variety of methods are available for the stabilization of proteins [6-8], from amino acid substitution to the simple addition of non-specific stabilising agents .

In the present paper we have investigated several simple and readily available techniques in an attempt to stabilize the enzyme against thermal inactivation at temperatures above 100°C, with a view to investigating the upper limit of enzyme stability.

At these temperatures, in addition to conformational unfolding, degradative reactions such as deamidation, hydrolysis, cysteine elimination, peptide bond hydrolysis, etc. will be important. All these reactions need water, so salts and polyols, for example, which diminish water activity, should stabilize the enzyme. To prevent unfolding of the protein, immobilization to solid matrices was tested, with and without additives. Chemical modification using glutaraldehyde was carried out to form new linkages within and between enzyme molecules which should also help to prevent the unfolding. Interprotein crosslinking with BSA was also tested.

Materials and Methods

Growth of bacteria:

Thermococcus strain AN1 was grown anaerobically under nitrogen without shaking in 2-litre Schott bottles as described by Hudson *et al.* [5], but the media contained 7g/l starch and 5g/l maltose. After 4 days incubation at 75 °C cells from 100 litres medium were harvested using a hollow fibre filtration system (Amicon, Danvers, MA, U.S.A.). The supernatant was concentrated through an Amicon S10 Y10 (10kDa) cartridge to a total volume of 8 l which was concentrated further through a PM 10 membrane to 1.6 l.

Purification of the α -glucosidase:

All column chromatography was carried out at room temperature. Fractions were concentrated and salt removed using Amicon PM 10 ultrafiltration membranes. The concentrated supernatant was applied in 400ml portions to a Hiload Q Sepharose 26/10 -column (10cm x 2.6cm; Pharmacia, Upsala, Sweden), equilibrated with 20mM bis Tris buffer, pH 6.3. The α -glucosidase activity was eluted at 0.3 M NaCl with a 560 ml linear 0-0.35 M salt gradient in the same buffer (flow rate was 4 ml/min). The pooled and active fractions were diluted in 0.1 M sodium phosphate-buffer pH 6.8 and applied to a Phenyl-Sepharose (low substitution) column (10cm x 5cm, Pharmacia). Activity was eluted with successive 200 ml volumes of 10, 15, 20, 25, 30, 40 and 50 % (v/v) ethyleneglycol. The ethyleneglycol was removed by repeated dilution with phosphate-buffer and ultrafiltration through PM10 Amicon membrane. Active fractions were applied to a Bio-Gel HPHT-hydroxyapatite chromatography column (10cm x 0.78cm) and eluted using a 40ml linear phosphate gradient of 10 - 350mM sodium phosphate, pH 6.8, at a flow rate of 0.5 ml/min; the α -glucosidase activity was eluted at about 30 mM phosphate. Active fractions were concentrated through Centricon cells (10 kDa, Amicon) to 1 ml and applied to a Superose P12 gel-filtration column (50cm x 1.5cm, Pharmacia) equilibrated with 20 mM sodium phosphate-buffer containing 0.2 M NaCl, pH 6.8 (flow rate 0.2 ml/min). The active fractions were finally applied to a Mono Q 5/5 (5cm x 1cm, Pharmacia) anion-exchange column equilibrated

with 20 mM bis-Tris buffer, pH 5.9. Activity was eluted at 0.15 M NaCl with a 180 ml linear 0- 0.4 M salt gradient at a flow rate of 0.6 ml/min into collection tubes containing 1 ml of 0.2M sodium phosphate buffer, pH 7.4, and 0.05% dithiothreitol.

For the stabilization studies enzyme preparations from the HPHT and Superose steps of the purification procedure were used.

Enzyme assay:

Activity was determined by measuring at 400 nm the pNP released from the pNP- α -D glucopyranoside by the enzyme at 75°C. The assay mixture, consisting of 4 mM substrate, 80 mM Mes buffer, pH 6.3, or 0.16 M sodium-phosphate buffer, pH 7.4, containing 0.04% (w/v) dithiothreitol and enzyme, in a total volume of 0.5ml, was incubated for an appropriate time at 75°C. The reaction was stopped by addition of 1 ml of 1 M sodium carbonate. One unit is defined as causing the release of 1 μ mol of pNP/ min (molar absorption coefficient of the substrate pNP = 18500 $\text{min}^{-1}\text{M}^{-1}$ at 400 nm).

Analysis of degradation products

Samples taken from assay mixtures were injected on to a h.p.l.c. Bio-Rad Aminex HPX-42A column. Filtered vacuum-degassed water formed the mobile phase at a flow rate of 0.6 ml/min, and a column temperature of 85°C. The products were monitored with a refractive-index detector. Standards used were 20 mM xylose, glucose, maltose, maltotriose, maltotetraose and maltohexaose, (standards and substrates were from Sigma, MO, USA). The substrate concentration was 40 mM in sodium phosphate buffer, pH 7.4, in the presence of 0.01% (w/v) dithiothreitol, and 3.5 mU of enzyme used per assay.

Thermostability determination

For the experiments below 100°C, the tests were carried out in eppendorf tubes fully immersed in the water during the incubation. For the experiments over 100°C, the tests were carried out in autosample vials (1.5/2ml, Alltech) in an oil bath. At intervals, duplicate tubes or vials were removed, cooled rapidly and stored on ice for the subsequent assay at 75°C.

SDS-PAGE and determination of pI

SDS-PAGE was performed on a Phast-System (Pharmacia) in a 10-15% (w/v) gradient polyacrylamide gel. Standard markers LMW (Pharmacia) from 14400 - 94000 Da were used. The PAGE conditions were these indicated by Pharmacia. Gels were stained by the Bio-Rad (Richmond, U.S.A.) silver staining procedure.

Isoelectric focusing was performed on a Phast System in polyacrylamide gels containing Pharmalytes 2-10 as described by Pharmacia. Standards were supplied from Serva (Heidelberg, FRG).

Stabilization techniques:**Cocrosslinking**

Porous protein particles were produced by aggregating proteins in the frozen state [10]: BSA (180 µg), enzyme (75µl of a 1 U/ml preparation), and glutaraldehyde (30 µl of a 50% solution) were mixed in 2.5 ml sodium phosphate buffer 0.2M, pH 7.4. After stirring, the mixture was incubated at -20°C for 2 hours, followed by 3 hours at 4°C. The sponge-like polymer was washed with 0.2M lysine and water and freeze-dried over night. The sponge-like polymer was homogenized to powder and 4 ml phosphate buffer was added..

Crosslinking [11,12]

Enzyme (75µl of a 1 U/ml preparation) and glutaraldehyde solution (50%, 200 µl) were stirred for 3 hours at room temperature in 2 ml 0.2 M sodium phosphate buffer, pH 7.4. The reaction mixture was then ultrafiltrated and diluted with buffer several times to remove the glutaraldehyde.

Immobilization :

Partially purified α -glucosidase was immobilized onto controlled-pore glass beads (pore diameter: 50nm, 104.9nm and 11.5nm with a particle size of 125 -177µ, 80-120µ and 80-120µ, respectively from Sigma, St.Louis, MO, USA), and onto long chain alkylamine-coated glass beads (pore diameter 50 nm, particle size 125-177 µ from Pierce, Rockford, USA; 0.33g) by the silane/glutaraldehyde coupling method [13 and

14, respectively]; and onto epoxy-beads (VA-Epoxy Biosynth Carrier from Riedel-de-Haen, Germany) [15] and oxirane acrylic beads (containing approx. 800 μmol of oxirane groups per g of solid 30 μm macroporous particles, from Sigma) by the method of Solomon *et al.* [16] with following modifications: Carrier (0.25 g) was incubated in 10 ml sodium bicarbonate buffer, 0.1M, pH 8.5, containing 0.05% (w/v) dithiothreitol with 300 μl enzyme solution (18 U/l preparation) for 48 hours at room temperatures with stirring, and one incubation mixture contained additionally 1% (w/v) BSA .

Linkage on polyethylenglycol was carried out according to the method of Beauchamp *et al.*[17], treatment in anhydrous organic solvent according to the method of Volkov [18] and linkage to new carbohydrate-based materials according to the method of Wang *et al.*[19]; Aminoglucose (430 mg) + 230 methacryloylchloride (90%, 230 μl) were incubated in 10 ml methanol at room temperature for 24 h. After centrifugation and extraction of the unreacted methacryloylchloride several times with methanol, the product was dissolved in 7 ml 0.2M tris buffer , pH 8, and 30 μl 45% ammonium persulphate was added. The mixture was degased (N_2) and incubated at 4 $^{\circ}\text{C}$ for 72 h. The polymer was ultrafiltered using an Amicon PM 1000 Da membrane and incubated with the enzyme in sodium borate buffer 0.2 M , pH 9, in presence of 0.1M NaBH_3CN at 37 $^{\circ}\text{C}$ for 5h, followed by an incubation overnight at room temperature. After filtration in a centricon cell (10 kDa), the solution was separated by gel filtration (Superose P12).

Results and Discussion

Enzyme purification

The α -glucosidase appeared to be extracellular; approximately 90% of the enzyme was found in the supernatant. The α -glucosidase was purified 875-fold with a yield of about 4% from the concentrated supernatant (table 1). The Superose P12 gel filtration was carried out at pH 6.8 in presence of 0.05 % dithiothreitol. When it was carried out at pH 6.3 and without dithiothreitol, the enzyme lost its activity overnight. The denaturation was irreversible, and changing the pH to 6.8 or 7.4, addition of 1% dithiothreitol, concentration of the sample, or the addition of 1% BSA did not recover the activity, although the enzyme was active in a less purified state for several months at pH 6.3. In the Mono Q step carried out at pH 5.9, eluted fractions were collected in tubes containing 1 ml of 0.2 M sodium phosphate buffer, pH 7.4, containing 0.05% (w/v) dithiothreitol to prevent irreversible denaturation.

The data in table 1 were determined in the absence of dithiothreitol. It was subsequently found that addition of dithiothreitol caused a large increase in activity (Table 3), which indicated the presence of oxidation-sensitive thiol group(s) on the enzyme. Given this, and the stimulation of the enzyme by BSA, it is possible that actual losses of enzyme during the purification are less than indicated by Table 3.

The SDS/PAGE of the final preparation revealed a single band of a molecular weight of 120 kDa, which was identified as the α -glucosidase by specific coloration of the band when incubating the gel in 40 mM pNP- α -D-glucopyranoside solution, pH 7.4, at 90°C for 20 min. Gel filtration on Superose 12 indicated a molecular mass of about 60 kDa. It is possible that hydrophobic interactions of the enzyme with the Superose column caused this low value. But incomplete unfolding of the protein on SDS treatment can cause a false molecular estimation also. As the enzyme is only readily denatured by SDS under conditions which might also lead to peptide bond hydrolysis (table 4), the molecular mass is not clear.

(After freeze-drying the enzyme and running a SDS/PAGE, one strong band appeared at 60 KDa.)

The molecular mass of most microbial α -glucosidases are below 70 kDa [2], however, α -glucosidases of high molecular mass (125, 130 and 140 kDa) have been reported in *Aspergillus awamori* [20] and in *Pyrococcus furiosus* [21] (125 kDa).

The isoelectric point was approximately 5.

pH optimum and pH stability

Stability tests of the purified enzyme at different pH showed that in a dilute solution and below pH 6.8 the enzyme is inactivated at room temperature after 48 hours. This denaturation could be prevented by addition of 1% (w/v) BSA in the incubation-mixture at pH 6.3, but not at pH 5.9 (Table 2). The enzyme in a less pure state is stable at pH 6.3 for several months.

The pH optimum is above pH 7 and no significant difference in activity was observed between pH 7 and 8.5. A slightly acid pH optimum is a feature of other α -glucosidases [2,22,23].

Inhibitors and activators

The activity was tested in presence of some bivalent metal ions and other additives (Table 3). The effect of ethanol can be attributed to transferase activity, the ethanol acting as a suitable acceptor for this reaction. Hg^{2+} , Co^{2+} , Ni^{2+} inhibit the enzyme activity, which might be expected given the effect of dithiothreitol, probably on thiol groups. Treatment with the chelator EDTA results in increasing the activity of the enzyme presumably by removal of trace heavy metals, while the bivalent metal ions Mg^{2+} , Ca^{2+} and Zn^{2+} slightly decrease the activity.

Table 4 shows that the enzyme is relatively unaffected by denaturing agents at 75°C. Activities without preincubation were similar.

Substrate specificity

The K_m of the glucosidase for pNP- α D-glucopyranoside is 0.41 mM using substrate concentrations of < 8 mM. V_{max} is 182 U/mg enzyme at 75°C in sodium phosphate buffer, 0.2M, pH 7.4, containing 0.01% (w/v) dithiothreitol.

The enzyme had no activity on any of following substrates (tested at 4 mM under standard assay conditions at 75°C and at pH 6.3): pNP- β -D-glucopyranoside, pNP- α -D-galactopyranoside, pNP- β -D-galactopyranoside, pNP- α -D-mannopyranoside, pNP- β -D-mannopyranoside, pNP- α -D-fucopyranoside, pNP- β -D-fucopyranoside, pNP- α -D-arabinopyranoside, pNP- β -D-xylopyranoside, pNP- β -D-lactopyranoside, pNP- α -D-rhamnopyranoside, pNP- α -D-maltoside, pNP- β -D-maltoside.

Analysis on the sugar-Pak HPLC column revealed that Isomaltose (Glc α 1-6Glc), Maltose (Glc α 1-4Glc), Nigerose (Glc α 1-3Glc), Palatinose (Glc α 1-6Fru), Turanose (Glc α 1-3Fru) and Panose (Glc α 1-6Glc α 1-4Glc) were hydrolysed. Table 5 shows the estimated specific activity on natural substrates compared to that on pNP- α -D-glucopyranoside. Surprisingly, the enzyme hydrolysed Panose, Palatinose and Nigerose at a higher rate than Maltose and Isomaltose.

No hydrolysis-products were detectable using 40mM maltotriose, maltotetraose, maltopentaose, maltohexaose, isomaltotriose, lactose (Gal β 1-4Glc), sucrose (Glc α 1- β Fru), raffinose (Gal α 1-6Glc α 1-2 β Fru), trehalose (Glc α - α' Glc), cellobiose (Glc β 1-4Glc), β -gentobiose (Glc β 1-6Glc), melizitose (Glc α 1-3 β Fru-2-1 α Glc), melibiose (Gal α 1-6Glc), or 1% (w/v) starch, amylose, dextran, amylopectin (α 1-4Glc α 1-6), pullulan (β 1-6maltotriose), inulin, or pectin after 50 hours incubation, using 0.34 μ g enzyme in 100 μ l at 75°C, pH 7.4.

Each sugar (concentrations up to 60 mM) was treated as a competitive inhibitor of pNP- α -D-glucopyranoside. The inhibitor constants K_i are shown in Table 5. Inhibitor concentrations were 30 and 60mM maltose, 60mM isomaltose, 30mM panose, 5mM nigerose, 60mM cellobiose, 30 and 60mM palatinose and turanose. Compared to the K_m for the substrate pNP- α -D-glucopyranoside the K_i values are relatively high for the substrates isomaltose (14 mM) and maltose (15.6 mM).

The values from the table 5 allowed the estimation of substrate preference, pNP- α -glucoside > nigerose (Glc α 1-3Glc) > palatinose (Glc α 1-6Fru) > panose (Glc α 1-6Glc α 1-4Glc) > isomaltose (Glc α 1-6Glc) > maltose (Glc α 1-4Glc) > turanose (Glc α 1-3Fru).

The α -glucosidase is distinct from other thermophilic α -glucosidases in its substrate specificity, showing a high hydrolytic activity towards pNPG and a preference order of α 1-3 > α > α 1-4 in glucose driers.

The thermostable α -glucosidase from *Clostridium thermohydrosulfuricum* 39E also showed higher rates of hydrolysis of panose and turanose than for maltose or isomaltose [24]. The purified α -glucosidase from *Pyrococcus furiosus* had highest activity towards maltose and isomaltose and lower activity toward pNP-glucoside [21].

Temperature effects

An Arrhenius plot of log (initial reaction velocity) against the reciprocal of temperature at pH 6.3 was linear below 105°C. The activation energy (E_a) of the enzyme as calculated from the Arrhenius plot is 73 kJ/mol.

Plots of log remaining activity against incubation time were linear. The half-lives of the enzyme at 98°C were $t_{1/2} < 40$ min in buffer; with added 1% (w/v) dithiothreitol $t_{1/2} < 150$ min; with 1% (w/v) BSA $t_{1/2} < 90$ min; and with both 1% (w/v) dithiothreitol and 1% (w/v) BSA $t_{1/2} < 215$ min, all in sodium phosphate buffer 0.2M, pH 7.4. These half-lives were dependant on the conditions employed (pH, ionic strength, enzyme concentration) and cannot be extrapolated to other conditions. Studies of the half-lives of the enzyme from the crude material gave values of > 60 min at 95°C and > 20 min at 103°C [24]. On purification, the enzyme became much less thermostable, although this seems to be due to oxidation and low protein concentration.

Enzyme stabilization

Enzyme was immobilized on to various matrices, including carbohydrates, and by crosslinking. In all cases except crosslinking (20% recovery) more than 40% of the original activity was recovered. Table 6 shows the K_m 's of the immobilized enzyme.

Of the various covalent modifications tried none enhanced stability significantly except linkage to carbohydrate-based material (Table 7).

A number of additives enhanced stability. Sorbitol was particularly successful, as found previously [], raising the half life by more than an order of magnitude (Table 7).

Dithiothreitol and BSA had even more dramatic effect, the former presumably by stabilising sulphhydryl groups; the effect of these with sorbitol was additive (Figure 1).

Although the carbohydrate-immobilized enzyme was the most stable of the covalent modifications, additives did not further enhance stability. The only case where the use of an additive with a covalently modified enzyme increased stability significantly over use of the additive alone was the epoxy-bead-linked (or oxirane acrylic bead-linked) and the cocrosslinked enzyme with 90% (w/v) sorbitol, which had half lives of 20-60 min at 130°C (Table 7, Fig.1 and 2). The stabilized enzyme exhibited the higher activity expected at 130°C compared with 100°C (Fig.3).

None of these techniques (Table 7) stabilized the enzyme at 140°C. In all cases all activity was lost after incubation of the enzyme for 5 min at 140°C.

Overall, it has been possible to raise the half-life of the α -glucosidase from about 5 min at 110°C to about 60 min at 130°C, by the additive effects of immobilization and sorbitol, corresponding to an increase in half life at a given temperature of between 2 and 3 orders of magnitude. This clearly indicates that not only is the enzyme conformationally stabilised at 130°C, but is also stabilized against degradation reactions: as suggested by Hensel [], conformational stabilization may confer resistance to degradation. In any event, there is no reason to suppose that the application of these techniques to more stable enzymes will not raise stabilities still further, nor that 130°C is the upper temperature limit for protein stability.

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Table 1: Purification of the α -glucosidase from *Thermococcus* strain AN1.

step	total activity	protein	spec. Activity	purification	yield
conc. supernatant	100 U	2500 mg	0.04 U/mg	1	100%
Hiload Q Sepharose	54 U	600 mg	0.09 U/mg	2.3	54%
Phenyl Sepharose	30 U	24 mg	1.25 U/mg	31	30%
HPHT	20 U	4.18 mg	4.8 U/mg	120	20%
Superose P12	11.5 U	0.4 mg	28.7 U/mg	718	11%
Mono Q	4 U	0.05mg	35 U/mg	875	4%

Activity was determined in Mes-buffer 0.1M, pH 6.3 using pNP α -glucoside as substrate at 75°C.

Table 2: Activity of the α -glucosidase in different buffers at pH 5.9, 6.3, and pH 6.8

buffer	A (19h)	B (48h)	C (48h,1% (w/v) BSA)
Mes 0.2M, pH 6.3	100%	0%	100%
bis Tris 0.2M, pH 6.3	91%	0%	100%
bis Tris 0.2M, pH 5.9	40%	0%	12%
sodium phosphate 0.2M pH 6.8	100%	100%	100%

Activity of the α -glucosidase in different buffers at pH 5.9, 6.3, and pH 6.8 was compared with the activity in presence of 1% (w/v) BSA. A: remaining α -glucosidase activity after prior incubation of the enzyme in buffer at room temperature during 19 hours; B: after 48 hours incubation, C: after 48 hours incubation in presence of 1% (w/v) BSA.

Table3: Activity of the α -glucosidase in presence of metal ions and other additives.

Additive	Mops buffer (0.2M, pH 7)
-	100%
DTT, 1% (w/v)	170%
BSA, 4% (w/v)	250%
EDTA, 1mM	146%
CaCl ₂ , 3mM	57%
MgCl ₂ , 3mM	87%
ZnCl ₂ , 3mM	37%
MnCl ₂ , 3mM	57%
HgCl ₂ , 3 mM	0%
FeCl ₂ , 1 mM	182%
CoCl ₂ , 3 mM	4%
NiCl ₂ , 3 mM	7%
CuCl ₂ , 3 mM	57%
CdCl ₂ , 3 mM	65%
Ethanol, 20%	196%

Enzyme was incubated in presence of the additives for 1 hour at room temperature prior to measuring the remaining activity. Triton-X-100 1%, NaCl 5%, AgCl 1mM, ammonium sulfate 2.4% (w/v) had no effect on the enzyme activity..

Table 4: Effect of denaturing agents on the enzyme activity

denaturing agent	1 hour preincubation
buffer, pH 7.4	100%
2% (w/v) SDS	116%
1% (w/v) SDS	121%
8M urea	43%
4M urea	73%

Activity of the enzyme in presence of urea and SDS in sodium phosphate buffer at pH 7.4 at 75°C. Preincubation was at room temperature.

Table 5: Specific activity of the enzyme and Inhibitor Constants.

Substrate	Specific activity	K_m or K_i
pNP- α -D-glucoside	22U/mg	0.41 mM
Nigerose	2 U/mg	2 mM
Panose	3.4 U/mg	13 mM
Palatinose	1.53 U/mg	11 mM
Isomaltose	1 U/mg	14 mM
Turanose	0.4 U/mg	17 mM
Maltose	0.26 U/mg	15.6 mM
Cellobiose	0 U/mg	30 mM

Table 5:

Enzyme specific activity was estimated by analysis of end products on a sugar PAK HPLC column.

Substrate concentration used were from 0.2 to 8 mM pNP- α -D-glucoopyranoside, and 40 mM for other substrates. Assays were carried out in sodium phosphate buffer 0.2 M, pH 7.4, containing 0.01% (w/v) dithiothreitol at 75°C. The enzyme concentration was 0.08 μ g per assay. Inhibitor concentrations of up to 60mM were used.

Table 6: Comparison of the K_m of the free and immobilized enzyme.

Immobilization on to	K_m
Free enzyme	0.41mM
Oxirane acrylic beads	0.56 mM
Epoxy-Carrier	3 mM
Alkylamino glass beads	8.6 mM
Uncoated controlled glass beads	8.2 mM
Co-crosslinked enzyme	0.71mM

K_m comparison of immobilized enzyme at pH 6.8, at 75°C with pNP- α -glucoside as substrate.

Table 7: Half-lives of the free and stabilized enzyme at 110, 120 and 130°C.

stabilization techniques	t _{1/2} at 110°C	t _{1/2} at 120°C	t _{1/2} at 130°C
Enzyme, Mes buffer, pH 6.3	~ 5	< 2	< 1
Enzyme, sodium phosphate buffer, pH 7.4	~ 5	< 2	
Glass Beads-enzyme (all sizes), pH 6.3	~ 5		
Alkylamine glass beads-enzyme, pH 6.3	~ 5		
Oxirane acrylic beads/ epoxy beads-enzyme, pH 6.3	~ 5		
Crosslinked Enzyme, pH 6.3 or pH 7.4	~ 3		
Co-cross linked enzyme, pH 6.3 or pH 7.4	~ 8		
PEG-enzyme, pH 6.3	~ 5		
Carbohydrate-enzyme, pH 6.3	~ 20		
13% ammonium sulfate, pH 6.3	~ 13	<2	
53% ammonium sulfate, pH 6.3	~ 165	~ 23	< 5
90% sorbitol, pH 6.3	~ 120	~ 60	< 5
99% sorbitol, pH 6.3		~ 60	
90% inositol, pH 6.3		~ 5	
50% trehalose, pH 6.3	~ 30		
90% trehalose, pH 6.3	> 60	~26	
90% glycerol, pH 6.3		~ 3	
90% sorbitol, pH 7.4	~415		~ 7
90% sorbitol, 1% DTT, pH 7.4	540		< 15
90% sorbitol, 1% BSA, pH 7.4	500		~ 30
90% sorbitol , 1% DTT, 1% BSA, pH 7.4	>>600		> 30
104.9nm glass beads+ 53% (NH ₄) ₂ SO ₄	>>100	~30	<2
104.9nm glass beads+ 90% sorbitol	>100	~18	<6
104.9nm glass beads + 50% trehalose	~70		
Alkylamino glass beads + 53% (NH ₄) ₂ SO ₄	~ 55	~ 25	
Alkylamino glass beads + 50 % trehalose	~ 35	~ 40	
Alkylamino glass beads + 90 % sorbitol	> 60	~ 60	
OAB, pH 7.4, + 90% Sorbitol			~ 20
OAB, pH 7.4, 90% sorbitol , 1% BSA			~ 30
OAB (linkage with E+BSA) , pH 7.4, 90% Sorbitol			~ 40
E+ 90% sorbitol, pH 6.8*	76	15 ~10(125°C)	< 2 (130°C)
Epoxy-carrier, pH 6.8, 90% sorbitol*	180	25 ~20 (125°C)	~ 6 (130°C)
Carbohydrate-Enzyme +90% sorbitol, pH 6.3		> 5	~ 5
PEG M20 or 4000-Enzyme+ 90% sorbitol, pH 6.3		~ 20	
Cocrosslinked enzyme +53% (NH ₄) ₂ SO ₄	~ 120		
Cocrosslinked enzyme + 50% trehalose	~ 90		
Cocrosslinked enzyme + 90% sorbitol, pH 6.3/7.4	> 120 (pH 6.3)	~ 90 (pH 6.3)	~ 60 (pH 7.4)

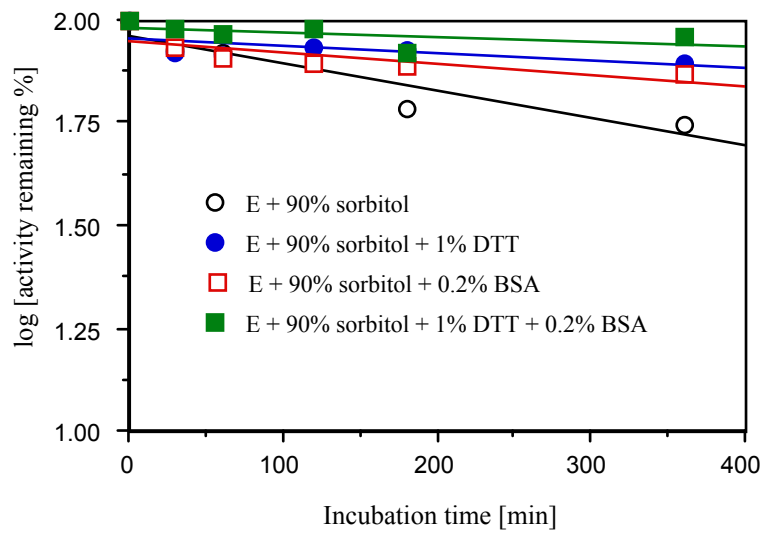
Table 8: half-lives at the indicated temperatures compared with the half-lives of the enzyme without stabilization. E; Enzyme, OAB: oxirane acrylic beads, PEG: polyethyleneglycol, DTT: dithiothreitol, % is % (w/v) unless otherwise mentioned.

Following additives did not enhance the stability at 110°C (or only very slightly):

99% (v/v) butanol, a mixture of butanol/water (7:3), of nonane/water (7:3), 10% polyvinylpyrrolidone 40 (~ 8 min), 20 % PEG M20 (~8 min) , 30% PEG 4000 (~ 10 min), 40% PEG 300 (~ 9 min), 1M NaCl (< 10 min), 10 % Triton X-100 (~5 min), 0.05 or 0.5% (v/v) DMSO (<5min), 90% (v/v) ethanol (<5min), 1% (w/v) BSA (< 8 min), 1% (w/v) DTT (< 8 min) , 1mM EDTA (< 5 min), 20% (v/v) ethanol (< 7 min), OAB enhance stability as much as epoxy beads, crosslinked enzyme + 90% sorbitol did not enhance stability further., Cocrosslinked enzyme contained 0.05% DTT.

Figure 1: Stability of the α -glucosidase at 110 and 130°C.

Stability at 110°C



Stability at 130°C

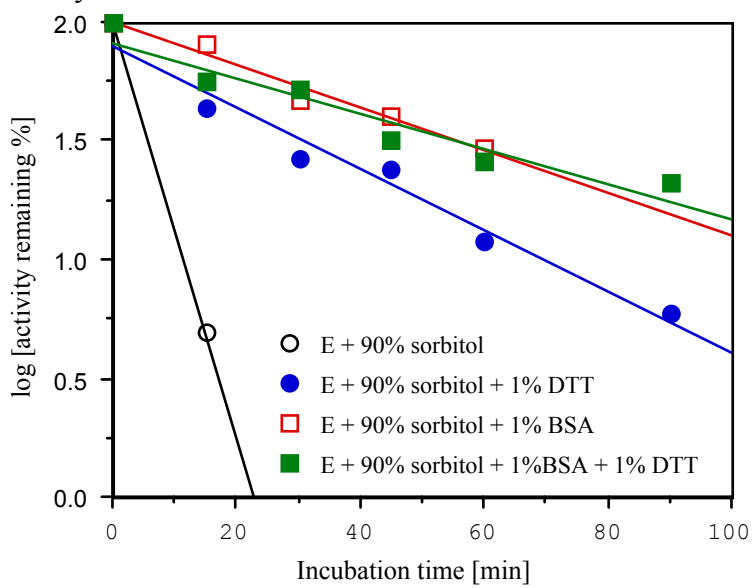


Figure 2: Stability of the free and epoxy-immobilized enzyme at 110, 125 and 130°C.

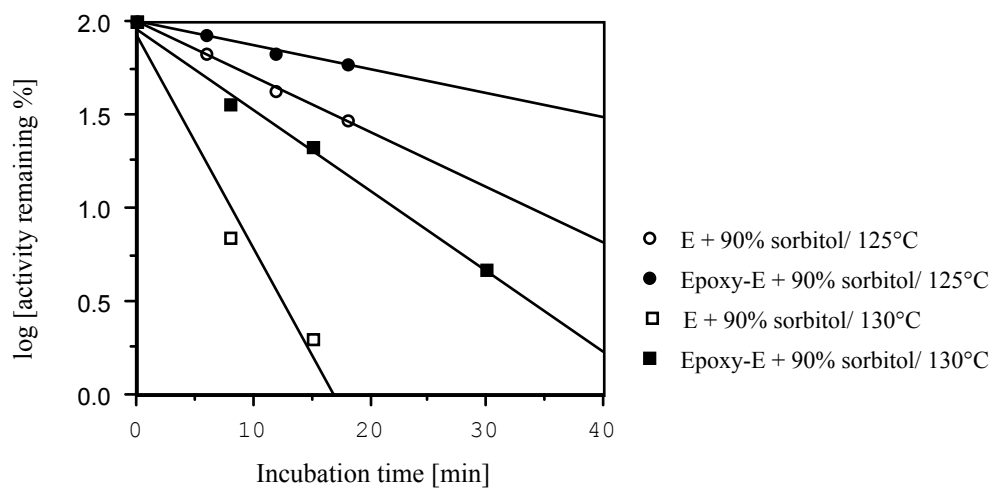
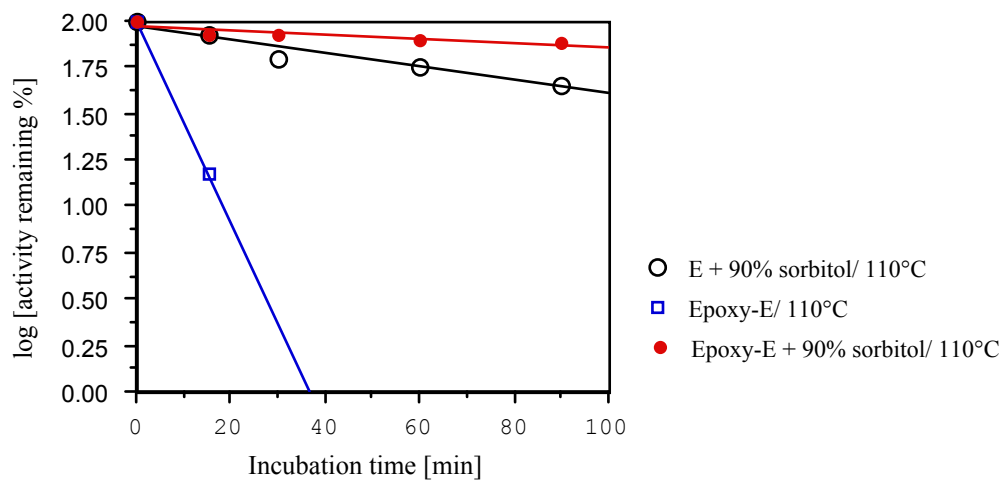


Figure 3: α -glucosidase activity at 110°C and at 130°C in sodium phosphate buffer, 0.2M, pH 7.4.

