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Properties of the recombinant α-glucosidase from *Sulfolobus solfataricus* in relation to starch processing

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Abstract

An α-glucosidase activity (EC 3.2.1.20) isolated from *Sulfolobus solfataricus* strain MT-4 was characterised and found of interest at industrial level in the saccharification step of hydrolysis process of starch. The gene encoding for the enzyme was expressed in *Escherichia coli* BL21 (DE3) with a yield of 87.5 U/g of wet biomass. The recombinant cytosolic enzyme was purified to homogeneity with a rapid purification procedure employing only steps of selective and progressive thermal precipitations with a final yield of 75.4% and a purification of 14.5-fold. The properties of this thermophilic α-glucosidase were compared with those of the α-glucosidase of a commercial preparation from *Aspergillus niger* used in the starch processing.

Keywords: α-Glucosidase; Starch process; Thermozymes; Enzyme purification

1. Introduction

In the last years, enzymes isolated from thermophiles have gained great attention because of their stability to high temperatures and towards common denaturant agents, and for the ability of working in conditions not suitable for mesophilic enzymes. However, the production of these enzymes from thermophiles is limited by the difficulty of growing large cultures of organisms at high temperature and by the high fermentation costs. This problem can be, however, overcome by heterologous expression of their isolated genes and many examples of efficient expression and recovery of functional enzymes are available [1–3]. In many instances, such proteins correctly fold in different environments at lower temperature. This strategy makes their production more competitive since it utilises conventional fermentation techniques and allows simple down-stream purification using denaturation steps. The starch industry, the second biggest consumer of enzyme sales,

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occupying 10–15% of the total world wide enzyme market [4], is one of the productive cycles that could benefit from biocatalysis to high temperatures since the production of glucose from starch is a multistage process, which involves different microbial enzymes in successive steps. In the first step, the starch is liquefied at 105°C for 5 min and later at 95°C for 90 min at pH 5.5–6.0 by a thermostable α-amylase. Subsequently, in the second step for dextrins saccharification, a α-glucosidase from *Aspergillus niger* is used, working optimally at pH and temperature of 4.5 and 60°C, respectively.

The starch conversion process could be improved by finding efficient and thermostable α-glucosidases acting at the pH and temperature of the liquefaction step. The α-glucosidase activity (EC 3.2.1.20) from the hyperthermophile *Sulfolobus solfataricus* strain MT-4 (*Ss*GA) can be of industrial interest since its thermophile properties could be used to improve glucose production from starch hydrolysates. To obtain suitable amounts of protein for application tests, the α-glucosidase gene was cloned and expressed in *Escherichia coli* and the protein purified to homogeneity (r*Ss*GA). Moreover, the activity of recombinant enzyme was compared to that of α-glucosidase from *A. niger* (*An*GA) present in the commercial enzyme preparation, dextrozyme, usually used in the saccharification step of starch process.

2. Experimental

2.1. Materials

2.1.1. Bacterial strains, plasmid, enzymes and chemicals

Cells of *E. coli* XL1-Blue and BL21(DE3) were purchased from Stratagene. Plasmid vector pT7-SCII was obtained from United States Biochemicals (USB). Restriction and modification enzymes were bought from Boehringher Mannheim (Milan, Italy) or Promega. T7 DNA-sequencing Kit was from Pharmacia. Dextrozyme (Novo) was used to study the α-glucosidase from *A. niger*. Glucose/GOD-Perid method Kit was purchased from Boehringher Mannheim.

All chemicals were from Sigma–Aldrich (Milan, Italy).

2.2. Methods

2.2.1. N-terminal sequence analysis

Partially purified α-glucosidase (200 μg) was loaded onto a 6% Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and after the electrophoretic run, the proteins were blotted on a PVDF filter (Immobilon P, Millipore) for 90 min at 120 V and 4°C. After staining with Coomassie Brillant Blue the high molecular weight band was cut and sequenced by Edman degradation on a Applied Biosystem 473A apparatus.

2.2.2. Plasmid construction

Routine DNA manipulations were carried out according to standard procedures [5].

The α-glucosidase open reading frame was isolated by PCR amplification using *S. solfataricus* (MT4) genomic DNA. The PCR mixture contained as primers 30 pmol of each oligonucleotides 5′-TCACAT**ATG**CAGACAATAAAAATATACGAG-AACAAAGG and 5′-CGCGG**ATC**CCTACTCTAG-GTTAATCTTTCCCCTAATTT introducing a *Nde*I site 5′ and a *Bam*HI site 3′ to the α-glucosidase gene, 100 ng of chromosomal DNA and 5 U of *Pfu* DNA polymerase (Stratagene) with 12 nmol of each deoxynucleotides triphosphate in 50 μl final volume. The temperature program was as follows: hot start, 5 min, 95°C, 10 cycles, 45 s, 95°C, 1 min, 48°C, 4 min, 72°C; 20 cycles, 45 s, 95°C, 1 min, 58°C, 4 min, 72°C; 1 cycle, 45 s, 95°C, 1 min, 58°C, 10 min, 72°C. The PCR product of approximately 2100 bp, was ligated to the *Nde*I and *Bam*HI cloning sites of the expression vector pT7-SCII obtaining the recombinant vector pT7αgly. In this construct, the α-glucosidase open reading frame is under the control of an IPTG-inducible promoter. The accuracy of amplification was checked on both strands by DNA sequencing by the dideoxy chain termination method [6]. The pT7αgly plasmid was transformed in the *E. coli* strain BL21(DE3). Recombinant cells were grown in Luria–Bertani medium [7] supplemented with ampicillin (100 μg/ml). Expression of the enzyme was induced by adding isopropyl-β-D-thiogalactoside (IPTG), 1 mM, to liquid cultures that reached an optical density at 600 nm of 0.6 and grown overnight.

2.2.3. Computer analysis

Sequences were compiled and analysed using DNA Strider™ 1.1. BLAST research engines were employed for sequence homology searches. ScanProsite Tool was used to find Prosite signatures.

2.2.4. Biomass production

Pre-cultures were grown in shaking flasks on a Luria–Bertani medium, the optical density was measured every hour, and when the absorbance at 600 nm was between 1.0 and 2.0, the culture was inoculated into the fermenter. The bioreactor used was a Biostat CT (2-l working volume), purchased from Braun Biotech International (Melsungen, Germany). The fermenter was equipped with a DCU for the control of pH, temperature, dissolved oxygen concentration, stirring and airflow rate. The pre-culture was diluted 1:10 into the fresh medium to start the batch experiment. The initial optical density was always between 0.1 and 0.2, corresponding to a biomass concentration of 0.17–0.34 g/l. The medium was prepared according to the recipe already used in the cultivation of *E.coli* K12 (Merkl) that contained: glycerol (40 g/l), citric acid (3 g/l), KH_2PO_4 (5.5 g/l), $K_2HPO_4 \times 3H_2O$ (5.6 g/l), $NaH_2PO_4 \times H_2O$ (2.2 g/l), $(NH_4)_2SO_4$ (1g/l), NH_4Cl (0.1 g/l), $MgCl_2 \times 6H_2O$ (1.2 g/l), $FeSO_4 \times 7H_2O$ (0.02 mg/l), $CaCl_2 \times 2H_2O$ (0.02 g/l), $MnSO_4 \times H_2O$ (12.9 mg/l), $ZnSO_4 \times 7H_2O$ (8.7 mg/l), $CoCl_2 \times 6H_2O$ (6.4 mg/l), $CuCl_2 \times H_2O$ (3.2 mg/l), $Na_2MoO_4 \times 2H_2O$ (2.7 mg/l), $AlCl_3$ (0.8 mg/l), H_3BO_4 (0.5 mg/l), and thiamine–HCl (5 mg/l). The mineral medium was supplemented with 2.4 g/l yeast extract and 1.2 g/l tryptone. The growth was followed by absorbance and weight measurements. When an optical density of 3.0 was achieved, 1 mM IPTG was added to the culture to induce the α-glucosidase production. The induction lasted 18 h. The final biomass yield was 25 g/l wet weight.

2.2.5. Enzyme assays

The enzymatic activity was usually monitored on *p*NPG. Enzyme solution (0.1–1 μg of r*Ss*GA), 0.1 ml, was added to 0.9 ml of 4.5 mM *p*NPG in sodium acetate, 50 mM, pH 5.5 (buffer A). The reaction mixture was incubated at 85°C for 5 min and stopped by adding 2 ml of 1 M sodium carbonate. The amount of *p*-nitrophenol released was measured by absorption at 400 nm in 1-cm cuvette, considering a molar extinction coefficient of 18,300 M^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme, which produces 1 μmol/min of *p*-nitrophenol [8].

α-Glucosidase activity was also determined using maltose as substrate. Fifty microliters of enzyme solution (0.25–1.67 μg of r*Ss*GA) were added to 5 mM of substrate in buffer A (final volume = 375 μl) in the same conditions of temperature. After 5 min, the reaction was stopped in an ice-water bath and the glucose produced was determined by the glucose/GOD-Perid method. A glucose standard curve ranging from 22 to 182 mg/l of glucose was performed. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol/min of glucose. *An*GA activity was monitored in the same conditions except that the temperature and pH were 60°C and 4.5, respectively.

Specific activity of r*Ss*GA on different maltodextrins (maltotriose, maltohexaose, amilose and starch) was also measured using glucose/GOD-Perid method. Fifty microliters of enzyme solution (0.25–1.67 μg of r*Ss*GA) was added to 1 mM maltotriose or 1 mM maltohexaose or 0.01% w/v amilose or 0.01% w/v starch in buffer A (final volume = 375 μl) in the same conditions of temperature.

2.2.6. Purification of recombinant SsGA

The cells, grown as mentioned above, were collected by centrifugation at $5000 \times g$ and the pellet was resuspended in Tris/HCl 10 mM pH 8 (3 ml buffer per each gram biomass), lysed by two French cell pressure treatments (2000 psi) and then centrifuged for 30 min at $10{,}000 \times g$ and 4°C.

The purification to homogeneity was achieved with five heat denaturation steps, which allowed the precipitation of almost all mesophilic host proteins:

The soluble fraction of the crude extract (10 ml from 6 g wet-cells) was subjected to thermoprecipitation under stirring for 30 min at 50°C, 60°C, 70°C and 80°C. After each treatment, the enzyme preparation was centrifuged for 30 min at $10{,}000 \times g$. In the last step, the supernatant was subjected to thermal precipitation under stirring for 24 h at 85°C.

The enzyme solution was then centrifuged for 30 min at 10,000 × *g*.

2.2.7. SDS–PAGE

SDS–PAGE under reducing conditions was performed on 6% acrylamide separating gel and 4% stacking gel according to Laemmli [9]. Ten to fifteen micrograms of protein were loaded on the gel and stained with Coomassie Blue R 250. High molecular weight markers (Pharmacia Biotech) were used as standard proteins.

2.2.8. Protein determination

The protein concentration was determined by the method of Bradford [10] with bovine serum albumin (BSA) as a standard protein.

2.2.9. Analysis of degradation products

Maltohexaose and glucose were identified by HPLC-DIONEX equipped with an electrochemical detector and a column specific for carbohydrates analysis named Carbopac PA 100. The chromatographic analysis was carried out using the following gradients: 160 mM NaOH (eluent A) and 300 mM AcONa + 160mM NaOH (eluent B), (time = 0–6 min, 0%, eluent A; time = 31 min, 100%, eluent B). For quantitative analysis, 11.6 μg/ml of maltoheptaose solution was added as internal standard.

2.2.10. Activity as function of pH and temperature

The activity of r*Ss*GA and *An*GA was determined according to standard procedure as a function of pH in 50 mM citrate–phosphate and Tris/HCl buffers in the pH range 3.5–6.5 and 7.5–8.5, respectively. r*Ss*GA and *An*GA activities were also monitored in the temperature ranges of 50–100°C and 15–80°C, respectively.

2.2.11. Enzyme stability

pH effect on r*Ss*GA stability was determined by incubating 1.0 U/ml of enzyme for 24 h at 4°C in 50 mM citrate–phosphate and Tris/HCl buffers in the range 3.5–6.5 and 7.5–8.5, respectively. Residual activity was determined in standard conditions. To estimate the thermostability of r*Ss*GA, the tests were carried out in eppendorf tubes fully immersed in water for 2 h at pH 5.5 at temperatures ranging from 50°C to 100°C, and then assayed under standard conditions. Furthermore, the r*Ss*GA was incubated at 75°C, 85°C and 100°C in order to determine the corresponding half-life. *An*GA thermostability was also estimated at 60°C, 75°C and 85°C. Duplicate test tubes were removed at different times, cooled rapidly and stored in ice. The residual activity was determined in standard conditions.

3. Results and discussion

3.1. Cloning and expression of SsGA

The genome of many thermophiles have been mapped and sized. Indeed, the recent elucidation of the complete genomic sequences for some hyperthermophiles provides the basis for identification of novel enzyme activities and also makes biocatalysts accessible. The N-terminal amino acid sequence MQTIKIYENKGVYKVVIGEP has been determined on the previously purified α-glucosidase from *S. solfataricus* strain MT-4 and was used to search its coding gene among the ORFs available from the *S. solfataricus* strain P2 genome sequencing project. The *SsGA* gene codes for a total of 693 amino acids with a calculated monomer molecular mass of 80.5 kDa, according to Rolfsmeier et al. [11,12]. The predicted gene product analysed with the BLASTP computer program [13], exhibits high similarity to several α-glycosidases, indicating that the protein belongs to the glycosil hydrolases protein family 31 [14,15]. These enzymes are classified as retaining because they cleave via a two steps mechanism the glycosidic bond with net retention of configuration. Three-dimensional structure of a member of this family is not currently available. The GIWLDMNEP motif of the family 31 active site was found in SsGA together with stretches sharing similarity to regions highly conserved among members of family 31. Aspartic residues in this region were shown to be essential for the catalytic activity of the sucrase–isomaltase complex [16] and of the human lysosomal α-glucosidase [17].

α-Glucosidases have been found in *Pyrococcus furiosus* [18] and in *Thermococcus* [19]. The first one is intracellular and shows a molecular mass of 125

Table 1
Purification procedure of recombinant glucoamylase starting from 6 g wet-cells of *E. coli* strain BL21(DE3)

	Volume (ml)	Total activity (U)	Total proteins (mg)	Specific activity (U/mg)	Activity yield (%)	Protein yield (%)	Purification factor
Crude extract	10	175	129	1.3			
Thermoprecipitation for 30 min at 50°C	9	152	85	1.8	86.8	66	1.4
Thermoprecipitation for 30 min at 60°C	8	151	38	4.0	86.3	29.5	3.0
Thermoprecipitation for 30 min at 70°C	7	151	18	8.4	86.3	14	6.5
Thermoprecipitation for 30 min at 80°C	6	144	12	12	82.3	9.3	9.2
Thermoprecipitation for 24 h at 85°C	5	132	7	18.8	75.4	5.4	14.5

kDa, while the second one is a monomeric extracellular enzyme with a molecular mass of 60 kDa. Both enzymes differ from SsGA regarding their structural properties. In fact, the *S. solfataricus* enzyme is a tetramer with 80 kDa subunits.

The *SsGA* gene was cloned into the pT7α gly plasmid, which was used to over-express the enzyme in *E. coli*. Crude extracts of IPTG-induced cells show a prominent new band on 6% SDS gels migrating at about 200 kDa. Only in recombinant induced cells, a thermostable and thermophilic α-glycosidase activity was detectable. Different growth media and lysis methods of cells were examined. For the highest yield of enzyme activity, 18-h induction with 1 mM of IPTG in the fermentation medium and French Press lysis, were used. r*Ss*GA activity was also assayed on the culture medium and cell membranes. While no activity was found in the broth, about 60% of total activity was retrieved on the cell membranes. The yield of r*Ss*GA obtained from *E. coli* after 1 mM IPTG induction was 87.5 U/g of wet-cells, while *Ss*GA activity is about 1.2 U/g of wet-cells. The units were calculated using maltose as substrate under standard conditions.

3.2. Purification of rSsGA

In general, methods employed for protein purification are time-consuming and expensive. However, when the gene of a protein from a hyperthermophile is expressed in a mesophile, a heat step is usually utilised for its purification, often followed by a column chromatography. In this work, however, r*Ss*GA was purified from *E. coli* cell extracts using only selective and progressive thermoprecipitation steps (Table 1), allowing removal of host denaturated proteins.

Following the heat treatments, 75.4% of the cytosolic r*Ss*GA was recovered, obtaining 7 mg of protein from 6 g of wet biomass. The enzyme, homogeneous by SDS–PAGE, shows a specific activity of 18.8 U/mg of proteins. The procedure reported here may be therefore of great interest for practical applications. The purification of r*Ss*GA in a single heat step was not possible because the denaturated *E. coli* proteins trap, by precipitation, the protein of interest in an irreversible manner.

3.3. Effects of pH on activity and stability of rSsGA and AnGA

The effects of pH on r*Ss*GA and *An*GA activities are shown in Fig. 1. The optimal pH of the r*Ss*GA

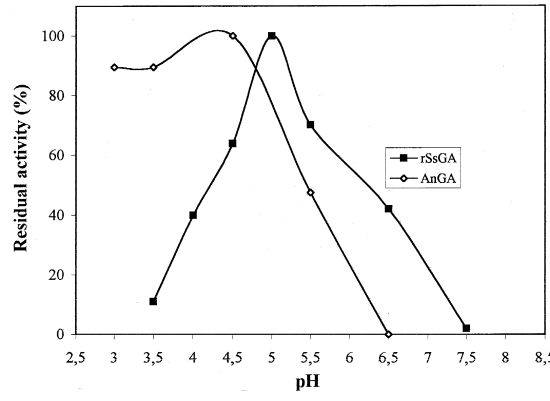

Fig. 1. Effects of pH on the activity of r*Ss*GA and *An*GA. The enzymes (1 and 0.5 U/ml, respectively) were assayed using *p*NPG as substrate under standard conditions in the following buffers: 50 mM citrate–phosphate buffer (pH 3.5–6.5) and 50 mM tris–HCl buffer (pH 7.5–8.5).

was 5.0. The enzyme exhibited a shift of 0.5 pH units towards the basic zone if compared to the α-glucosidase from *S. solfataricus* 98/2 (4.5 to 5.0) [11]. The value of optimal pH of r*Ss*GA was also different from that one observed in *S. shibatae* DMS 5389 (5.5) [20]. The optimal pH of *An*GA was 4.5.

Both enzymes share the same stability profile, preserving 100% of the initial activity from 3.5 to 8.5.

The optimal pH feature of r*Ss*GA makes it suitable to industrial applications. In fact, salts that are generally added to the starch mixture to adjust pH must later be removed by exchange resins. These additions and removals make the production process more complex, increasing time, costs and environmental pollution. The use of the recombinant enzyme in the saccharification step, avoiding the lowering of pH, would allow glucose production without these disadvantages.

3.4. Temperature effects

The optimal temperature of r*Ss*GA was 100°C similar to that determined by Rolfsmeier et al. [12]. r*Ss*GA enzyme showed a great stability to high temperatures. After 2-h incubation at various temperatures no loss of activity up to 85°C was observed. The 78.8% and 44.8% of initial activity was measured at 90°C and 100°C, respectively (Fig. 2). The

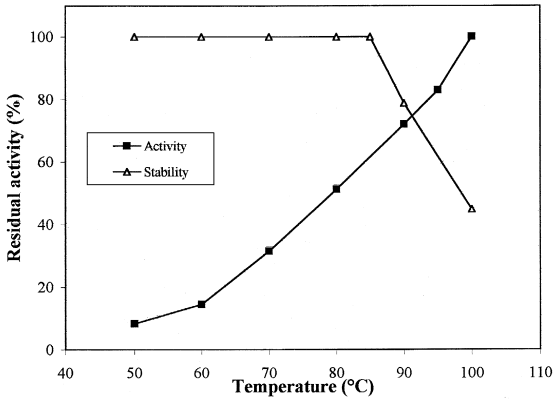

Fig. 2. Effects of temperature on the stability of r*Ss*GA. The enzyme (1 U/ml) was incubated at different temperatures using *p*NPG as substrate under standard conditions. Thermostability was determined by incubating the enzyme (1 U/ml) at pH 5.5 for 120 min at different temperatures and measuring the residual activity at 85°C.

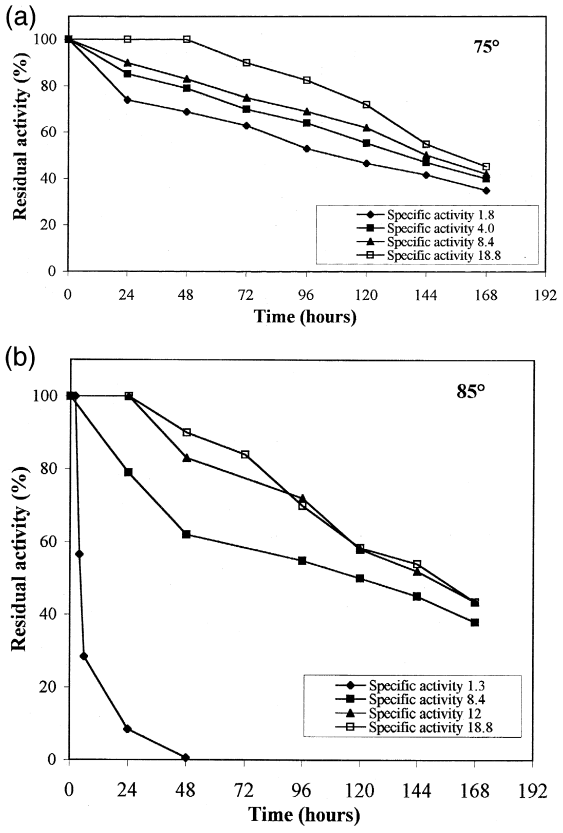

Fig. 3. Effects of temperature on the stability of r*Ss*GA at 75°C (a) and 85°C (b) at different specific activities. The enzyme (1 U/ml) was incubated at pH 5.5 and residual activity was determined using *p*NPG as substrate under standard conditions.

thermostability of r*Ss*GA enzyme at 75°C and 85°C are shown in Fig. 3a and b, respectively. The results showed that the stability of recombinant enzyme in solution was conditioned by the purity of enzymatic preparation because of the co-precipitation of r*Ss*GA and *E. coli* proteins. Nevertheless, at 75°C, all enzyme solutions preserved more than 50% of initial activity in the typical time range of saccharification step (from 40 to 70 h). The same behaviour was observed when the enzyme solutions were incubated at 85°C, except for the crude extract where most of initial activity was quickly trapped by denaturated proteins of *E. coli*. The thermotolerance of enzyme at 100°C was also determined (Fig. 4). The half-lives of the purified enzyme at 75°C, 85°C and 100°C were 156, 150 and 1.20 h, respectively.

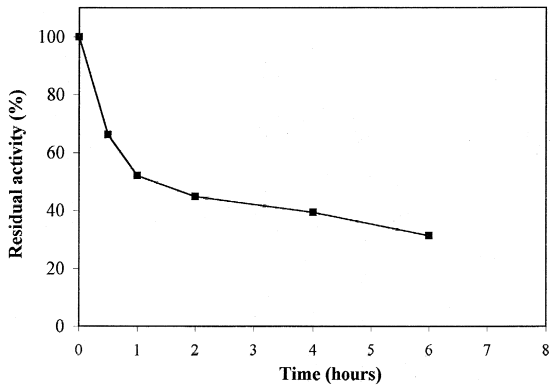

Fig. 4. Effects of temperature on the stability of r*Ss*GA at 100°C. The enzyme (1 U/ml) was incubated at pH 5.5 for different times and residual activity at different times was assayed using *p*NPG as substrate at 85°C.

The optimal temperature value of *An*GA was 65°C. The half-life at 60°C was 63.4 h, while at 75°C and 85°C, the enzyme activity was detected for only a few minutes.

The α-glucosidase from *S. solfataricus* is notably more stable if compared to that one from *A. niger* at all temperatures investigated. This property makes it more competitive in the saccharification process by avoiding a drastic cooling of the system. Moreover, since the recombinant enzyme can operate for extended time at high temperatures, a higher substrate solubility and the sterility of bioreactor are guaranteed during the whole process.

3.5. Substrate specificity of rSsGA

Among the substrates assayed (*p*NPG, maltose, maltotriose and maltohexaose), the purified enzyme had the greatest preference for maltose. The enzyme was totally inactive on amilose and starch (Table 2). These properties limit its use only in the saccharification process.

3.6. Maltodextrins hydrolysis

The starch process, which is actually the major source of glucose and fructose in the world, is realized in two phases reported above. The entire process has a number of adjustments to pH, solid concentration and temperature. The immediate opportunities of the industry are quite clear. The short-term goal could be the introduction of a biocatalyst that lower the overall cost of glucose production from starch. Bearing this in mind, some properties of r*Ss*GA like optimum temperature (100°C), optimum pH (5.0) and high stability at 75°C and 85°C make such enzyme of industrial interest since it could be used to improve glucose production from starch hy-

Table 2
Substrate specificity of r*Ss*GA. Assay conditions were described in methods

Substrate[a]	Specific activity (μmol of glucose min^{-1} mg^{-1})
*p*NPG	18.8 ± 1.0
Maltose	35.6 ± 1.0
Maltotriose	6.95 ± 0.5
Maltohexaose	6.66 ± 0.5
Amylose	–
Starch	–

[a] Substrate concentrations were 4.5 mM for pNPG, 5 mM for maltose, 1 mM for maltotriose and maltohexaose and 0.01% (wt/v) for amylose and starch.

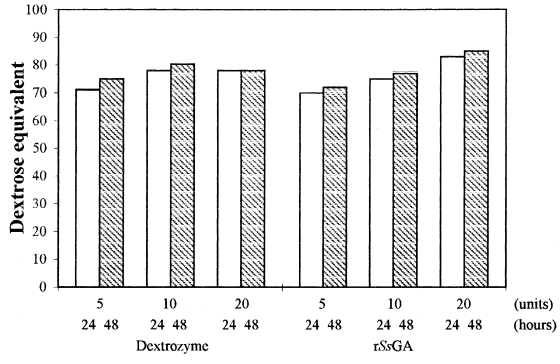

Fig. 5. A reaction mixture containing 30% maltohexaose in 0.05 M sodium acetate buffer was incubated using 5, 10 and 20 units of *An*GA and r*Ss*GA. The process parameters were 60°C and pH 4.5 for *An*GA (Dextrozyme) and 75°C and pH 5.5 for r*Ss*GA. In order to estimate the amount of glucose produced, samples were withdrawn after 24 and 48 h and analysed by a Dionex HPLC system. Enzyme units used in the reaction mixture were measured using maltose as substrate.

drolysates. In order to estimate the rate of hydrolysis of maltodextrins at industrial concentrations and compare the properties of r*Ss*GA and *An*GA, 30% of maltohexaose as substrate was used. To simplify the experiments on a laboratory scale, the hydrolysis of maltohexaose was carried out in batch using enzyme solutions. In order to obtain the maximum amount possible of glucose 5, 10 or 20 units of each α-glucosidase were used. The operation parameters were 60°C at pH 4.5 for *An*GA (Dextrozyme) and 75°C at pH 5.5 for r*Ss*GA. Both enzymes were able to perform an hydrolysis with Dextrose equivalent (DE) value of about 70 already after 24 h. After 48 h, the production of glucose increased up to about 80 DE with 10 and 20 units for both enzymes (Fig. 5). At the end of the reaction, 20% w/v of initial substrate was recovered as maltohexaose and shorter maltodextrins. Small differences in the substrate hydrolysis were found when different amounts of enzyme units were tested. For its thermal properties, r*Ss*GA can be used at temperatures higher than 60°C, reducing the cooling energy of reactor. Moreover, as the whole starch process could be carried out at pH 5.5 as in the liquefaction step, the decrease of pH could be avoided.

4. Conclusions

The characteristics of recombinant α-glucosidase from *S. solfataricus* permit the design of a starch conversion process without changing pH and drastically reducing temperature in the saccharification step, thus lowering significantly the cost of sugar production. In addition, the high expression level (87.5 U/g wet biomass) together with the easy downstream process (heat treatments) make r*Ss*GA of special interest for industrial application. Further experiments are in progress in order to optimise the bioreactor configuration and process conditions.

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