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ORIGINAL PAPER

Physiological aggregation of maltodextrin phosphorylase from *Pyrococcus furiosus* and its application in a process of batch starch degradation to α-D-glucose-1-phosphate

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Abstract Maltodextrin phosphorylase from *Pyrococcus furiosus* (PF1535) was fused with the cellulose-binding domain of *Clostridium cellulovorans* serving as an aggregation module. After molecular cloning of the corresponding gene fusion construct and controlled expression in *Escherichia coli* BL21, 83% of total maltodextrin phosphorylase activity (0.24 U/mg of dry cell weight) was displayed in active inclusion bodies. These active inclusion bodies were easily isolated by nonionic detergent treatment and directly used for maltodextrin conversion to α-D-glucose-1-phosphate in a repetitive batch mode. Only 10% of enzyme activity was lost after ten conversion cycles at optimum conditions.

Keywords Thermophilic enzyme · Maltodextrin phosphorylase · *Pyrococcus furiosus* · Recombinant enzyme · Physiological aggregation

Introduction

Starch can be a cheap source of sugars for derivatizing protein to produce glycoproteins, a potential new class of pharmaceutics or food additives [1]. Degradation of soluble starch by maltodextrin phosphorylase (EC 2.4.1.1) represents a more effective way of activated sugar monomer production compared to the polymer degradation to maltose and glucose with subsequent phosphorylation. Phosphoactivated sugars derived from α-D-glucose-1-phosphate (Glc1P) can be used directly for glycosylation by non-Leloir glycosyltransferases, otherwise they have to be transferred to a nucleotide for their subsequent use by Leloir glycosyltransferases (after Luis F. Leloir, the scientist, who discovered the first sugar nucleotide and who received the 1970 Nobel Prize in Chemistry).

Potato maltodextrin phosphorylase (MP) [2] and *E. coli* MP [3] have been the first enzymes employed for Glc1P production. Both enzymes show excellent stability—2 months for potato MP [2] or 600 h for *E. coli* MP [3]. However, they had to be purified from other contaminating starch and Glc1P hydrolyzing enzymes. In the case of recombinant *E. coli* MP, a removal of trace phosphatase amounts cleaving Glc1P was essential and this could be achieved neither by affinity precipitation with starch, or precipitation with Bioprocessing Agent BAP 1050 (Toso Haas, Stuttgart, Germany), nor by ion-exchange chromatography (Q-Sepharose, Pharmacia) or anion-exchange membrane filtration (CIDE 1000, CIQM 1000, Millipore) [3]. Later, thermostable MPs from *Thermus caldophilus* [4, 5] and *Thermoanaerobacter tengcongensis* [6] were explored for Glc1P production. In addition to the obvious potential advantage of higher process temperature to promote better solubilization and disorganization of the raw materials [7], thermostable enzymes can be overexpressed in a mesophilic host whose enzymes are completely inactivated during the thermal reaction.

An accumulation of insoluble aggregates, widely known as inclusion bodies (IBs), is a common observation during recombinant protein production in prokaryotic hosts (especially expression of proteins from thermophiles). IBs of recombinant enzymes were long considered as a nonproductive protein waste. However, a number of studies revealed (for a recent review, see [8]) that the proteins deposited in the IBs have acquired native-like secondary structure, suggesting that their aggregation is not random.

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Consequently, enzymes trapped in the IBs are not necessarily inactive, as demonstrated in several cases [9–11]. Moreover, it was recently shown that upon recombinant enzyme fusion with an aggregation module (CBD_{clos}), the activity of chimeric protein can be almost completely "pull-down" into active inclusion bodies (aIBs) [12].

Pyrococcus furiosus starch metabolism has been already studied and PF1535 gene was confirmed to encode an enzyme with MP activity showing specificity to maltotetraose or longer maltodextrins [13]. However, other biochemical properties of *P. furiosus* MP (*Pf*MP) are not known. This work is aimed to explore technological properties of *Pf*MP *N*-terminally fused with CBD_{clos} (CBD_{clos}–*Pf*MP).

Materials and methods

Materials

HotStarTaq® Master Mix Kit, QIAquick PCR Purification Kit, QIAprep Spin Miniprep Kit were from QIAGEN (Hilden, Germany). The linearized plasmid vector pET-34b(+) and T4 DNA Polymerase, that is suitable for ligation-independent cloning, were from Novagen (Madison, WI). *P. furiosus* genomic DNA was obtained from American Type Culture Collection (ATCC 43587D). Primers were from Invitrogen (Paisley, Scotland). CelLytic™ B Cell Lysis Reagent (Sigma, B7435-500 ML) was used for aIBs preparation.

As substrate, dextrin from maize starch (Fluka, 51636-100G-F) was used. All other reagents were of analytical grade from Sigma-Aldrich (St Louis, MO).

Cloning, expression and isolation of aIBs

PF1535 gene was amplified from *P. furiosus* genomic DNA in 50 µl PCR reaction using forward 5′GACGACGAC AAGTTGGTTAAAGTGGAGAAT3′ and reverse 5′GAG GAGAAGCCCGGTTACTCTTTCTTTTCAAT3′ primers. The target gene with LIC extensions was purified, treated with T4 DNA polymerase for preparation of overhangs, and annealed with the linearized vector.

Freshly transformed *E. coli* BL21(DE3) cells harboring the recombinant plasmid were grown overnight (30 °C, 225 rpm) in 30 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) supplemented with kanamycin (30 µg/ml). Then 10 ml of the culture was transferred into fresh LB (100 ml) medium containing kanamycin and grown at 37 °C. When A_{600} value reached 0.9–1.0, temperature was decreased to 25 °C, the agitation rate was reduced to 100 rpm, and after addition of 400 µM isopropyl-1-thio-*β*-D-galactopyranoside (IPTG), allowed to proceed for 20 h.

The cells were harvested by centrifugation (4,500*g*, 10 min, 4 °C) and lysed with ten volumes of the non-ionic lytic detergent. After centrifugation of the lysate (20,000*g*, 10 min, 4 °C), the debris was washed three times with 25 volumes of buffer (50 mM Tris–HCl, pH 7.5). The soluble and insoluble fractions were assayed for proteins (Lowry, BSA as a standard) and enzyme activity as described below.

Dextrin transformation into Glc1P

The reactions were performed in 1.5 ml (Eppendorf Safe-Lock) tubes placed in a thermomixer (Eppendorf, Thermomixer comfort).

For activity comparison of soluble and insoluble fractions, 10 mg of lyophilized cells were lysed in 0.5 ml lytic detergent and insoluble fraction was suspended in 0.5 ml Tris buffer (50 mM, pH 7.5). Both fractions were mixed with 0.5 ml reaction mixture: 10% dextrin, 500 mM KH_2PO_4, and pH was adjusted to 7 with KOH. Then, the reaction was performed at 90 °C and 10 µl aliquots were taken and frozen at 10 min intervals.

One milliliter of reaction mixture for each point of optimal dextrin, P_i concentration, pH, or thermal activity was evaluated in triplicate.

In the case of P_i optimization, 1 ml reaction mixture contained 10% dextrin (m/V), 100–1,000 mM KH_2PO_4 and 700 µg CBD_{clos}–*Pf*MP, or in the case of optimization of dextrin content, 1 ml reaction mixture contained 1–10% (m/V) dextrin, 400 mM KH_2PO_4 and 700 µg CBD_{clos}–*Pf*MP. The reactions were performed overnight at pH 7 and 90 °C.

pH was optimized in 1 ml reaction mixture that contained 5% (m/V) dextrin, 400 mM KH_2PO_4 and 700 µg CBD_{clos}–*Pf*MP. The pH was adjusted by KOH. The reaction ran overnight at 90 °C. Thermal activity was explored in the same reaction mixture but pH was adjusted to 8 and the initial rate was measured (10 µl aliquots were taken and frozen at 20 min intervals in the first 2 h).

When the optimal conditions (5% dextrin, 400 mM KH_2PO_4, pH 8, 85 °C) were established, then 3.5 mg CBD_{clos}–*Pf*MP was suspended in 1 ml reaction mixture and time-course was followed for 2 h (frozen 10 µl aliquots) in triplicate. After 2 h, the tubes were centrifuged (5 min., 35,000*g*) and sediments were resuspended in a new reaction mixture. Two to three conversion cycles were done per day and the samples were then kept at 4 °C.

Glc1P concentration level determination

A volume of 50 µl of appropriately diluted sample was mixed with 50 µl of a solution containing 5 mM UTP and 10 mM $MgCl_2$. After thermal equilibration at 25 °C, 14 U (5 µl) of UDP–glucose pyrophosphorylase (GalU, EC 2.7.7.9) was added and the reaction was allowed to proceed for 15 min, and then the samples were frozen. The yield of

UDP–glucose was measured by HPLC. The chromatography was performed on a Shimadzu system (LC-10AD, SPD-10AV) equipped with a TESSEK Separon SGX NH_2 column (150 × 3.3 mm i.d.; 7 µm) using an isocratic elution with 50 mM H_3PO_4, 10 mM $MgCl_2$ (pH 6.4, triethylamine) at a flow rate of 0.5 ml/min.

Results and discussion

Choice of maltodextrin phosphorylase and its fusion to an aggregation module

P. furiosus is an obligately anaerobic, heterotrophic, hyperthermophilic archaeon that was isolated from a shallow hydrothermal vent near Vulcano Island, Italy [14]. The organism grows optimally near 100 °C and utilizes starch as primary carbon source. The transcriptional analyses showed that the main pathway for the starch utilization involves only three enzymes, amylopullulanase (PF1935), maltodextrin phosphorylase (PF1535), and 4-α-glucanotransferase (PF0272). These enzymes generate glucose-1-phosphate and glucose, which both then enter into the glycolytic pathway of this organism [13]. This would indicate that the maltodextrin phosphorylase (MP) could be effective and hyper-thermostable which is ideal for an industrial use of an enzyme degrading starch. We cloned PF1535 gene into pET-34b plasmid and overexpressed a chimeric protein composed of an aggregation module (CBD_{clos}) [12] and *N*-terminally fused MP (CBD_{clos}–*Pf*MP). SDS-PAGE analysis of soluble and insoluble cell fractions (Fig. 1) showed that the target protein of expected molecular mass 118 kDa was displayed in insoluble fraction, and thereby CBD_{clos}–*Pf*MP was precipitated into IBs. However, this protein was partially degraded and a band of *Pf*MP (a predicted molecular mass of 98 kDa) was detected in both fractions prepared from lysed *E. coli* cells. HPLC-based analysis of the fractions revealed that the soluble fraction consisted of 17% and the insoluble fraction consisted of 83% of the whole cell MP activity. It means that a larger part of MP cannot leak out from the cell and the whole *E. coli* cells or their debris pellets could be industrially used in a repetitive batch conversion of dextrin to Glc1P.

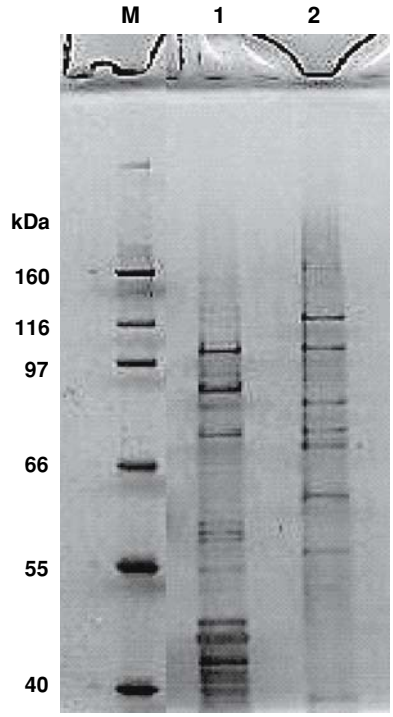

Fig. 1 SDS-PAGE: *Lane M* marker proteins whose molecular masses are indicated on the *left*; *lane 1* soluble fraction of the cells expressing CBD_{clos}–*Pf*MP; *lane 2* insoluble fraction of the cells expressing CBD_{clos}–*Pf*MP. The expected molecular masses of CBD_{clos}–*Pf*MP and *Pf*MP are 118 and 98 kDa, respectively

Influence of substrate concentration, pH, and temperature on the conversion of dextrin to Glc1P

In phosphorolysis, either inorganic phosphate or the glucan substrate may be a limiting component determining the maximum achievable concentration of the product, Glc1P. In the presence of a molar excess of α-glucan (maltooligosaccharides or starch), the Glc1P yield is limited mainly by the initial concentration of inorganic phosphate. Therefore, a ratio of substrate to orthophosphate is needed to be optimized in the reaction system before scaling-up the process [4]. Figure 2 depicts influence of orthophosphate (P_i) concentration on Glc1P yield in the presence of 10% maltodextrin. High initial phosphate concentration had a negative effect. A concentration of 400 mM P_i was determined to be an optimal value. The amount of Glc1P in the equilibrium linearly decreased with the initial P_i (Fig. 2, inset). Maximal Glc1P yield (24%) is in accordance with an equilibrium constant ($K_{eq} = 0.22$–0.24) calculated for *E. coli* maltodextrin phosphorylase [3]. The second experimental set optimized α-glucan concentration in the presence of 400 mM P_i (Fig. 2). An α-glucan limitation was observed when the maltodextrin concentration was below 5%.

The thermophilic MPs from *Thermococcus litoralis* [15], *Bacillus stearothermophilus* [16], *Thermotoga maritima* [17] showed optimal pH below 7 (pH 6.5–7.0) contrary to mesophilic *E. coli* MP which has an optimum at 7.5 [3]. It should be emphasized that pH optimum of the initial rate differ from pH optimum of the highest equilibrium yield, for example *E. coli* MP has the maximal equilibrium yield at pH 8.0 [3]. We focused on batch conversions; therefore, an influence of pH on the equilibrium yield was analyzed (Fig. 3). The optimal Glc1P yield occurred at pH

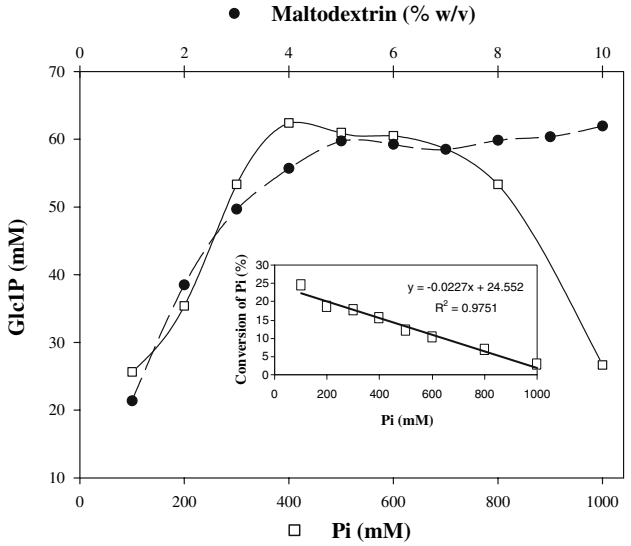

Fig. 2 Equilibrium in dependence on inorganic phosphate (*open square*) and α-glucan (*filled circle*) concentration. The reaction mixture contained 10% dextrin, 100–1,000 mM KH_2PO_4 and 700 μg CBD_{clos}–*Pf*MP in the case optimization of inorganic phosphate content, and 1–10% dextrin, 400 mM KH_2PO_4 and 700 μg CBD_{clos}–*Pf*MP in the case optimization of α-glucan content. The reactions were performed at pH 7.0 and 90 °C. *Inset* conversion of P_i (%, mM/mM)

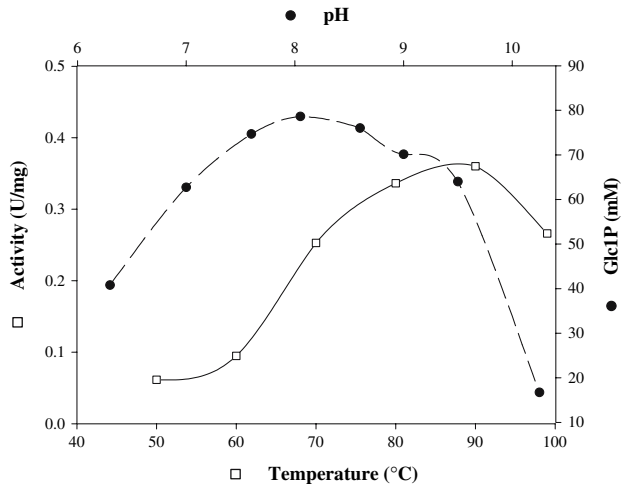

Fig. 3 pH-Dependent equilibrium of the phosphorolysis (*filled circle*) and temperature effect on the activity of CBD_{clos}–*Pf*MP (*open square*). pH was optimized at 90 °C in a reaction mixture that contained 5% dextrin, 400 mM KH_2PO_4 and 700 μg CBD_{clos}–*Pf*MP. Thermal profile of activity was explored in the same reaction mixture while pH was adjusted to 8

8.0, which is similar to the optimum equilibrium pH for the *E. coli* MP [3].

Temperature optimum was measured as an initial rate in the range of 50–99 °C (Fig. 3). Enzyme activity increased from 50 °C and reached a maximum at 90 °C. A maximum activity level of 73% was observed at 99 °C. There was only a moderate increase of activity between 80 and 90 °C. From the view of long-term stability, temperature of 85 °C was chosen as working temperature.

Performance of insoluble cell fraction during repeated batch operation

The stability of the aggregated maltodextrin phosphorylase from *P. furiosus* during the conversion of dextrin to Glc1P was evaluated by using the insoluble cell fraction during ten batch operation cycles (Fig. 4). Conditions established in previous experiments were applied (400 mM P_i, 5% maltodextrin, 85 °C, pH 8). Each conversion cycle took 2 h and Fig. 4 depicts the time-course of the first one. The precipitated phosphorylase showed good stability, loosing approximately 10% of the activity after reaction cycles. A new stock solution of the reaction mixture was used in the 7th cycle. One can hypothesize that this moderate loss in the yield could be caused by this change of the reaction mixture.

Conclusions

PF1535 gene was successfully cloned and fused with an aggregation module. The active form of the modular protein was completely entrapped in the insoluble fraction. A part of the overexpressed protein was cleaved, and due to this reason, a non-tagged soluble phosphorylase was present in the cytosol. However, the activity which leaked out from the cell debris or whole cells represented only 17%. A total of 83% of activity remained in aIBs that can be repetitively used for at least ten conversion cycles. The 83% deposition of MP into aIBs corresponds to 0.197 U per mg

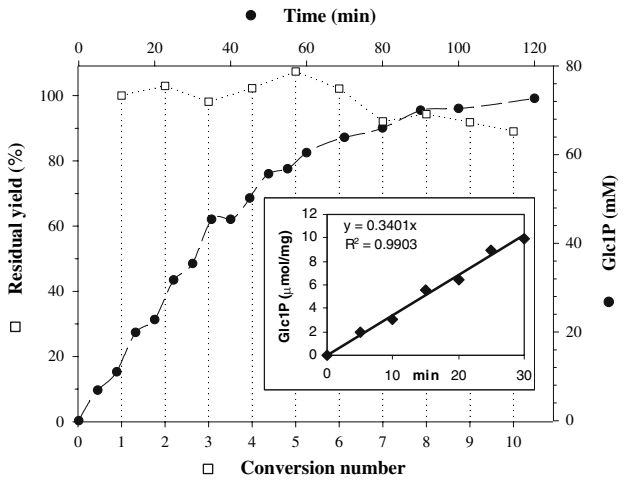

Fig. 4 Repeated batch conversion with recycled insoluble cell fraction (*open square*). Time course of the first conversion (*filled circle*). *Inset* calculated activity per mg of protein for the initial 30 min period. Conditions: 400 mM P_i, 5% maltodextrin, 85 °C, pH 8

of dry cell weight (DCW) since the recombinant cells tolerated 0.237 U/mg of DCW. Since *E. coli* reaches a density of 6 g of DCW/l of culture broth during the growth in a bioreactor, up to 1,182 U of MP may be trapped into the aIBs obtained from 1-l culture. Such a volumetric activity is comparable with that of recombinant *E. coli* maltodextrin phosporylase produced in a 7-l MBR fermenter (1,100 U/l) [3]. In a special fed-batch cultivation, the recombinant mesophilic host, engineered for production of a thermophilic enzyme, was able to grow to a density of 15 g DCW/l [18]. This type of cultivation could result in volumetric activity of 2,955 U/l. These values together with high thermal stability favor CBD_{clos}–*Pf*MP for an industrial degradation of starch. However, MP has low K_{eq} for Glc1P/P_i, which probably will convince one who is considering production of Glc1P as a final product, to choose more expensive sucrose and sucrose phosphorylase due to its much higher K_{eq}. For example, a stable conversion of 600 mM P_i and 600 mM sucrose to 550 mM Glc1P was reached recently with the use of sucrose phosphorylase from *Leuconostoc mesenteroides* [19]. This enzyme also reached much higher volumetric activity of 60,000 U/l in a bioreactor [19].

On the other hand, aggregated maltodextrin phosphorylase need not be isolated because the mesophilic phosphatases are thermally inactivated. CBD_{clos}–*Pf*MP could be perfectly applied in a repetitive batch process where a coupled enzyme continuously depletes produced Glc1P. For example, when we have coupled GDP–mannose phosphorylase from *P. furiosus* (PF0589) [20], then 99% of phosphate was converted to Glc1P.

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