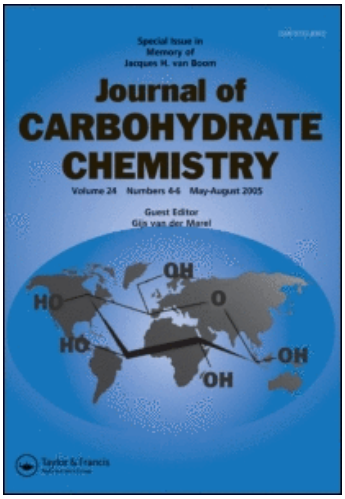

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Enzymatic Synthesis of α-Glucose-1-phosphate: A Study Employing a New α-1,4 Glucan Phosphorylase from *Corynebacterium callunae*[1]

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ENZYMATIC SYNTHESIS OF α-GLUCOSE-1-PHOSPHATE: A STUDY EMPLOYING A NEW α-1,4 GLUCAN PHOSPHORYLASE FROM *CORYNEBACTERIUM CALLUNAE*[1]

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ABSTRACT

In synthetic pathways to complex carbohydrates such as oligosaccharides or nucleotide sugars the activated sugar 1-phosphates serve as important starting molecules. In this study the enzymatic synthesis of α-glucose-1-phosphate (Glc-1-P) has been investigated using a new bacterial α-glucan phosphorylase from *Corynebacterium callunae*. The major factors governing the rate of reaction and the attainable degree of substrate conversion have been identified and, accordingly, for optimizing the yield and limiting reaction time for the enzymatic process several points must be considered: (i) the pH-dependent equilibrium of reaction, (ii) product inhibition of the phosphorylase and (iii) enzymatic cleavage of α-1,6 glycosidic linkages present in α-1,4-glucans such as starch or maltodextrins by pullulanases to improve their phosphorolytic conversion. Results obtained in continuous experiments with the phosphorylase retained in an ultrafiltration membrane reactor confirmed the complete operational stability of the enzyme for several days at 30 °C. Since no more than approximately 18 % of the inorganic phosphate can be converted into Glc-1-P an efficient procedure for phosphate and product recovery will be particularly important.

INTRODUCTION

The initial step of storage polysaccharide mobilization and utilization in eukaryotic and prokaryotic organisms is catalyzed by α-1,4-glucan phosphorylases (EC 2.4.1.1) according to:

$$(\alpha\text{-1,4-glucoside})_n + P_i \Leftrightarrow (\alpha\text{-1,4-glucoside})_{n-1} + \alpha\text{-D-Glc-1-P}$$

The reaction product, α-glucose-1-phosphate (Glc-1-P), has conserved to a large extent the energy of the cleaved glycosidic linkages of the polymer in its phosphate-ester bond. Glc-1-P is a compound found in all living matter and has several interesting properties and, consequently, several fields for practical application are known. Glc-1-P may be used as antiinflammatory and immunosuppressive or cytostatic agent in medicine[2] but also serves as an important intermediate for the synthesis of more complex carbohydrates such as oligosaccharides[3-6] or nucleotide sugars.[7-9] Furthermore, selective chemical oxidation of 6-CH_2OH in Glc-1-P on noble metal catalysts is rather easily possible and yields glucuronic acid-1-phosphate (GlcA-1-P).[10] This compound is expected to be a useful intermediate for the synthesis of glucuronic acid (after enzymatic cleavage of GlcA-1-P by phosphatase), glucaric acid or ascorbic acid.[10-12] In these or other areas, quite surprisingly, not many reports are found in the literature dealing with possible applications of α-glucan phosphorylases, which are a biochemically well characterized group of enzymes having pyridoxal-5' phosphate as an enzyme bound cofactor.[13] This possibly means that the synthetic potential of these phosphorylases may not yet have been fully exploited.

In view of employing α-glucan phosphorylases for the synthesis of Glc-1-P we were especially interested in the bacterial phosphorylases which lack complex regulation on the enzyme level and which could be efficiently produced by fermentation. Among the bacterial enzymes only the maltodextrin and glycogen phosphorylase from *Escherichia coli* and an α-1,4-glucan phosphorylase from *Klebsiella pneumoniae* have yet been characterized in detail.[14-17] Recently we have reported the identification, isolation and partial characterization of a new α-glucan phosphorylase in Gram-positive *Corynebacterium callunae*.[18] This enzyme has been used for the production of Glc-1-P from several α-glucans and it has been found that substrate pretreatment by enzymes cleaving α-1,6 glycosidic linkages can considerably enhance the product yield.[18-20] The present report deals with further studies on improving the enzyme production by *C. callunae* and on identifying the major factors limiting rate and the final degree of substrate conversion in the phosphorolytic reaction catalyzed by this enzyme.

RESULTS AND DISCUSSION

Enzyme Production. As shown recently fermenter cultivations of *C. callunae* on a complex growth medium yielded approximately 25 $g \cdot L^{-1}$ wet biomass with a phosphorylase activity of 8-10 $U \cdot g^{-1}$ biomass when a roughly optimized concentration of 16 $g \cdot L^{-1}$ maltodextrin is employed. In view of reducing media costs for enzyme production, *C. callunae* growth and concomitant synthesis of maltodextrin phosphorylase

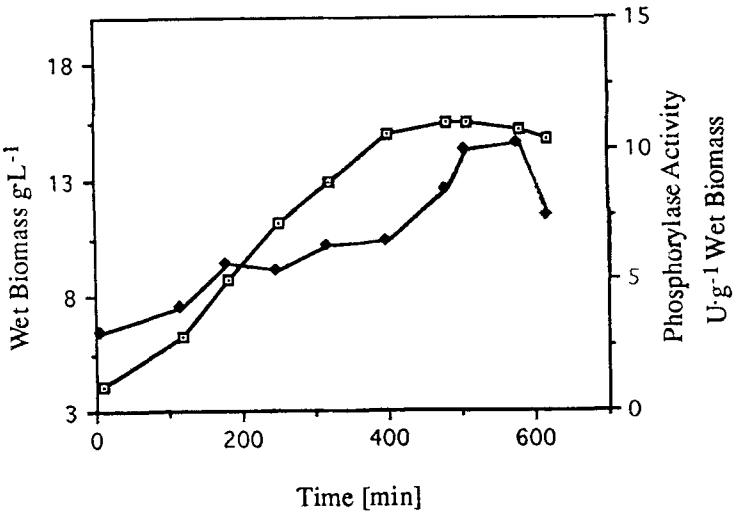

Figure 1. Time course of a bioreactor cultivation of *C. callunae* on mineral medium and of the concomitant phosphorylase synthesis by the organism (wet biomass, —□—; phosphorylase, —◆—).

was studied on mineral medium supplemented with 16 $g \cdot L^{-1}$ maltodextrin. The corresponding time course of a bioprocess experiment on a 10 L scale is shown in Figure 1. While the yield of biomass is approximately 30-40 % lower than on complex medium, the specific phosphorylase activities ($U \cdot g^{-1}$ biomass) are identical on both media. Further improvement of the enzyme production could thus be expected from cultivating *C. callunae* to higher cell densities than reported here (>100 $g \cdot L^{-1}$)[21] while retaining a constant specific phosphorylase activity. This is currently investigated in discontinuous and fed-batch cultivations using glucose and maltodextrin as a mixed carbon source.

Enzyme Purification. For the production of Glc-1-P the phosphorylase preparation must contain neither phosphatase nor phosphoglucomutase activity and it is of imperative importance to achieve this minimal degree of purity. The presence of periplasmic, specific Glc-1-Phosphatase has been reported in *E. coli* [22] and the negative effect of a contamination by this or another phosphatase for the synthesis of Glc-1-P by the maltodextrin phosphorylase from *E. coli* was clearly demonstrated.[19] For the *C. callunae* enzyme an adequate 2-step purification procedure using ammonium sulfate precipitation followed by hydrophobic interaction chromatography on Phenylsepharose has recently been established by Weinhäusel et al.[18] An interesting alternative though appeared to be precipitation with weak cationic polymers (BPA 1050) and subsequent

Table 1. Purification of phosphorylase from *C. callunae*

Scheme A			Scheme B		
	Spec. Activity U/mg	Yield %		Spec. Activity U/mg	Yield %
cell extract	0.23	100	cell extract	0.24	100
BPA precipitation[a]	0.78	87	Ammonium sulfate precipitation[b]	0.20	60
Q-Sepharose	4.3	65	Phenylsepharose	5.3	57

a 5.000 ppm at pH 5.3 and 6 mS; b 25 % saturation

use of ion exchange chromatography. A comparison of both strategies is shown in Table 1. The purification scheme B carried out according to Weinhäusel et al.[18] gave slightly lower enzyme yields but higher specific activities than earlier reported, most probably because of deviating fermentation conditions in this study. As found here with both purification schemes phosphorylase preparations with well comparable specific enzyme activities in almost identical yields were obtained (Table 1). It was observed, however, that storage of the enzyme in the presence of BPA 1050 led to a 50 % loss of activity within two months. Since both enzyme preparations did not contain detectable contaminating activities, they appear to be well suited for Glc-1-P production. In the presence of 50-70 mmol·L^{-1} inorganic phosphate the partially purified enzyme preparations were perfectly stable for up to 100 days at 25 and 4 °C.

Kinetic Properties. Kinetic characterization of *C. callunae* phosphorylase revealed that Michaelis Menten constants for both substrates, phosphate and glucan, are strongly dependent on the buffer composition. Especially for the case of phosphate the K_m value of the enzyme may vary by up to one order of magnitude from 1 to 20 mmol·L^{-1} in respectively Tris- or phosphate buffer. Because a phosphate-buffered solution seems ideal for the synthesis of Glc-1-P and pH values between 7.0 - 8.0 have recently been reported optimal,[18] the respective kinetic constants have been determined under these conditions (Table 2.). While the catalytic rates with either maltodextrins or soluble starch are identical, the affinity of the phosphorylase for the latter substrate and the resulting catalytic efficiency are approximately 3 times higher.

A possible inhibition of the phosphorylase by its product Glc-1-P is difficult to accurately quantify especially at high concentrations of Glc-1-P. Using 300 mmol·L^{-1} phosphate and 10 g·L^{-1} maltodextrin the rate-retarding effect of Glc-1-P was however

Table 2. Kinetic constants of *C. callunae* phosphorylase at 30 °C and pH 7.5 under conditions for production of Glc-1-P.

Substrate	K_m	Activity[a]	rel. catalytic efficiency	R^2
	$g \cdot L^{-1}$	$V_{max,rel}$	(Activity / K_m)	
Maltodextrin	9.20	1.17	0.127	0.99
Soluble Starch	3.31	1.18	0.356	0.97

a related to 1 U in the standard assay (Experimental); R^2 is the coefficient of determination

clearly seen with an almost complete suppression (90 %) of the phosphorylase activity in the phosphorolysis direction already at a Glc-1-P concentration of 40 - 50 $mmol \cdot L^{-1}$. It can be argued, however, whether these data can be interpreted in terms of enzyme inhibition because Glc-1-P also serves as a substrate in the reverse direction of reaction i.e. the synthesis of glucan.

Factors Limiting Conversion. The major factor determining the maximum conversion of inorganic phosphate into Glc-1-P by α-glucan phosphorylase is the pH-dependent equilibrium of reaction. The equilibrium concentration of Glc-1-P is correlated in a nearly linear manner with the initial concentration of the hydrogen phosphate ion (HPO_4^{2-}) which could thus be considered as being the reactive phosphate ion. At 30 °C approximately 18 % of the total initial phosphate can be converted (Figure 2) whereas the (non limiting) concentration of α-glucan has a minor influence. Since both Glc-1-P and possibly the linear as well as the branched limit dextrins inhibit the phosphorylase catalyzed reaction, equilibration may require incubation times of several days when high phosphate concentrations (> 500 $mmol \cdot L^{-1}$) are employed.

When compared with the enzyme sucrose phosphorylase (EC 2.4.1.7) which is capable of almost fully utilizing inorganic phosphate for the synthesis of Glc-1-P and fructose from sucrose,[23] the values found here are quite low. Therefore, it will be necessary to recyle non-reacted substrates (especially phosphate) and to establish a not yet available efficient procedure for product recovery.[24]

The maximum conversion of different types of α-glucans is typically between 30 and 50 % which can at least in part be attributed to α-1,6-glycosidic linkages present to a varying extent in different α-glucans. Consequently, the pretreatment of the α-glucans by pullulanases which are capable of cleaving the α-1,6-glycosidic bonds in the substrate may enhance this percentage of conversion considerably. For optimizing the pretreating reaction the catalytic action of pullulanase was followed measuring both the time-

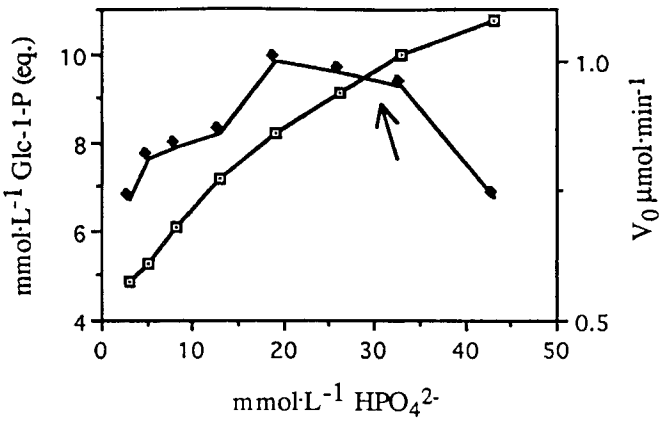

Figure 2. Effect of hydrogen phosphate concentration (which is in turn determined by the pH value) on the reaction equilibrium (—▫—) and on the initial rate (—◆—) of the phosphorylase catalyzed conversion of 50 mmol·L^{-1} inorganic phosphate and 20 g·L^{-1} maltodextrin at 30 °C. The arrow indicates the conditions chosen for Glc-1-P production corresponding to a pH value of 7.5.

dependent release of reducing sugars as well as formation of iodine-starch complexes. Both manners of quantification correlate very well with the attainable degree of substrate conversion in the subsequently initiated phosphorolysis reaction[19] (Figure 3).

The time-courses of maltodextrin degradation by either *C. callunae* phosphorylase alone or by pullulanase and phosphorylase acting simultaneously were compared. While there is apparently no difference in the initial rate of Glc-1-P formation, the product yield after exhaustive reaction is up to 1.5 fold higher in the presence of pullulanase (Figure 4). As a consequence, when a high conversion of the α-glucan into Glc-1-P is desired, utilization of pullulanase will be important. With approximately 100 - 250 units pullulanase per gram glucan being employed, the resulting conversions of the α-glucan by the phosphorylase in a simultaneous or a sequential enzyme action are well comparable.

In view of synthesizing defined low molecular weight, linear maltooligomers from the heterogeneous maltodextrin substrate as the limit dextrins of the combined pullulanase and phosphorylase action, analysis of the residual oligosaccharide after incubation with pullulanase and phosphorylase was carried out by thin-layer chromatography on silica plates.[26] It was found that neither a homogeneous nor an exclusively linear maltooligosaccharide is formed as the end-product upon this combined enzymatic attack on the technical maltodextrin substrate. With respect to maximizing the productivity of the enzymatic process for Glc-1-P synthesis, it can be disputed whether

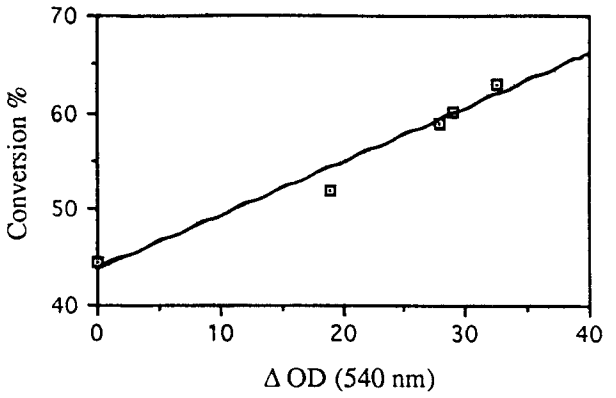

Figure 3. Effect of pullulanase pretreatment (120 pullulanase units (PUN) per gram glucan and 50 °C) on the subsequent maximum conversion of 10 g·L^{-1} maltodextrin (30 °C, 300 mmol·L^{-1} phosphate, pH 7.5) by *C. callunae* phosphorylase. The efficiency of pullulanase action is measured as release of reducing sugars.[25]

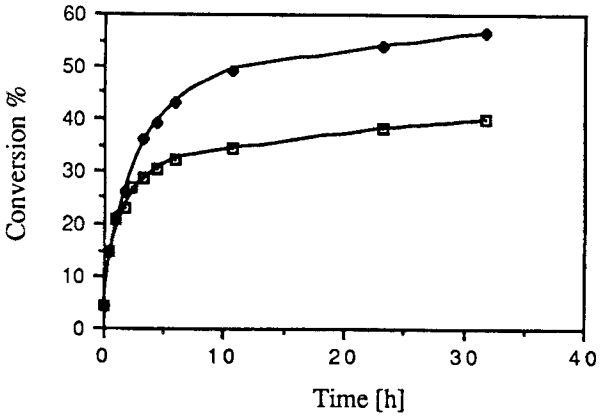

Figure 4. Conversion of 10 g·L^{-1} maltodextrin by 1 U·mL^{-1} phosphorylase in 300 mmol·L^{-1} phosphate buffer pH 7.5 in the presence (—◆—) or absence (—□—) of 120 U pullulanase per gram α-glucan.

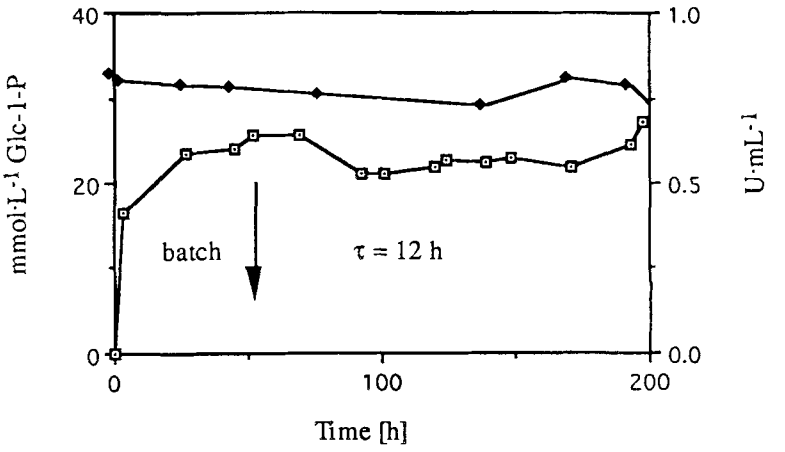

Figure 5. Continuous conversion of 10 g·L^{-1} non treated maltodextrin in 300 mmol·L^{-1} phosphate buffer pH 7.5 (glucan limit) by *C. callunae* phosphorylase (—□—, Glc-1-P; —◆—, enzyme activity). τ is the average residence time.

substrate pretreatment by pullulanase can have a large beneficial effect because a significant decrease of the reaction rate is already observed between 30 - 40 % conversion (Figure 4).

Continuous Conversions. Continuous experiments retaining the enzymes by conventional ultrafiltration membranes were carried out with the aim of elucidating the operational stability of the *C. callunae* phosphorylase for further optimizing the productivity of the enzymatic process. The conversion profile obtained with non-treated maltodextrin employing glucan-limited conditions is shown in Figure 5. An equilibrium of substrate fed and Glc-1-P formed is observed for the course of several reactor cycles and indicates no appreciable loss of enzyme activity and productivity within almost two weeks of reaction. The operational stability of the enzyme is corroborated by measurements of the time-dependent phosphorylase activity in the reactor. Studied either in glucan-limit (Figure 5) or in phosphate-limit the results obtained in continuous experiments largely confirmed the data obtained in discontinuous mode of operation. As for not yet clear reasons the exception to that is the pretreatment of the maltodextrin by pullulanase in a simultaneous manner with the phosphorylase action. The conversions found were always significantly (5-10 %) lower than expected from the results of batch experiments. The ultrafiltration membrane completely retains both the phosphorylase and the pullulanase and also the stability of the latter enzymes under reaction conditions does not seem to account for the insufficient pretreatment of the substrate.

In view of optimizing the productivities in the continuous reactor one important conclusion seems to be that the enzymatic process cannot be operated at conditions close to the reaction "equilibrium", i.e., close to the maximum attainable degrees of phosphate or glucan conversion. The reason is that the rates of reaction are decreased significantly under these conditions because of product inhibition by limit dextrins and possibly Glc-1-P, depletion of the α-glucan (cf. Figure 4) and possibly a reduced enzyme activity on small oligosaccharides.

The novel enzyme from *C. callunae* seems to be well suited for the application in the continuous process described since it can be produced and purified in sufficient quantities and high yields and also exhibits excellent stability under reaction conditions. At least the complete conversion of the inorganic phosphate into Glc-1-P is thermodynamically impossible without continuously removing the product during reaction. Also the productivities decrease rapidly in the presence of Glc-1-P. The development of an efficient method for separating Glc-1-P and non-reacted phosphate as well as glucan will thus be of utmost importance for the enzymatic process with respect to both product recovery but also substrate recycling. A combination of electrodialysis and ion exchange chromatography is currently investigated and gave encouraging preliminary results.

EXPERIMENTAL

Enzyme Production. Precultures of *Corynebacterium callunae* DSM 20147 were obtained from cultivations of the organism for 12 hours in 1 L baffled Erlenmeyer flasks (30 °C, 180 rpm) with 300 mL basal growth medium according to Weinhäusel et al.[18] Bioprocess experiments were carried out in a stirred tank reactor (MBR, Wetzikon, Switzerland) with a working volume of 10 L. The fermenter medium contained the following components ($g \cdot L^{-1}$ of bidistilled water): 16 maltodextrin (Cerestar, Vilvoorde, Belgium), 5 meat extract, 0.4 $MgSO_4 \cdot 7H_2O$, 0.002 $FeSO_4 \cdot 7H_2O$, 0.002 $MnSO_4 \cdot 4H_2O$, 0.100 $CaCl_2 \cdot H_2O$, 20 NH_4Cl, 0.5 KH_2PO_4 and 1.0 K_2HPO_4. The pH of media solutions was adjusted to 7.2 prior to sterilization. The controlled cultivation conditions were: 30 °C, stirrer speed of 650 rpm, aeration rate of 1.5 vvm (pO_2 at 40 % saturation), constant pH of 7.4. Antifoam (polypropyleneglycol) and alkali (3 $mol \cdot L^{-1}$ potassium hydroxide) were automatically added as required. After inoculation with 1L of preculture samples (250 mL) were taken periodically, and crude cell extracts were prepared as described below and analyzed for phosphorylase activity.

Enzyme purification. Cells of *C. callunae* were harvested at 6.000 rpm in a ZK 401 centrifuge (rotor 6.9; Hermle, Gosheim, Germany) for 20 min at 4°C. Cells were resuspended (1:4) in buffer (50 $mmol \cdot L^{-1}$ potassium phosphate, 10 $mmol \cdot L^{-1}$ EDTA,

7μmol·L^{-1} mercaptoethanol, pH 6.9), and disrupted in a Dyno Mill (Bachofen, Basel, Switzerland; 0.1-0.2 mm beads) while maintaining the temperature inside the cell at about 4 °C. Crude cell extracts were obtained thereafter by ultracentrifugation (30.000 rpm, 40 min, 4 °C) in a Beckman L-70 ultracentrifuge (Beckman, Fullerton, CA; rotor 45.1 TI).

After (i) ammonium sulfate precipitation (25 % saturation) at pH 6.8 or (ii) precipitation with Biocryl BPA 1050 (Toso Haas, Stuttgart, Germany) at pH 5.3 the supernatant was cleared by ultracentrifugation (30.000 rpm, 20 min, 4 °C) at 30.000 rpm (rotor 70.1 TI). Further purification of the supernatant was done in case (i) by hydrophobic interaction chromatography on an FPLC system (Pharmacia; Uppsala, Sweden) using an XK50 column of phenyl-sepharose fast flow (Pharmacia) eluting with a linear gradient of ammonium sulfate (20-0 % w/v) in buffer (50 mmol·L^{-1} K_2HPO_4, pH 6.9). In case (ii) anion exchange chromatography on a Q-Sepharose column (Pharmacia) equilibrated with 50 mM phosphate buffer pH 6.9 was used eluting with a linear gradient of 0.25 - 0.50 mol·L^{-1} NaCl in the equilibration buffer. Eluted protein was monitored at 280 nm. Protein solutions were concentrated in 30 kDa cut-off ultrafiltration tubes (Centriprep; Millipore, Bedford, MA) at 4 °C and 1.500 x g.

Assays and Analytical. Phosphorylase activity was quantified in a coupled assay employing phosphoglucomutase and glucose-6-phosphate dehydrogenase as described by Weinhäusel et al.[18] using maltodextrin DE 19.4 (Agrana, Vienna, Austria) as a standard substrate. One unit (U) of enzyme activity refers to 1 μmol NADH produced per min at 30 °C. Accordingly, Glc-1-P was enzymatically measured. Phosphatase activity was measured by reported methods[28] using either *p*-nitrophenyl-phosphate or Glc-1-P as a substrate. Protein was determined using the Bradford dye binding assay[29]. Inorganic phosphate was measured by the Spectroquant assay (Merck, Darmstadt, Germany)

Discontinuous and Continuous Substrate Conversions. The initial rate kinetics (discontinuous measurement[18]) and the time course of substrate degradation catalyzed by phosphorylase were determined at 30 °C in phosphate buffered solution at pH 7.5 while varying the type of glucan and its concentration. Also the molarity of the buffer was varied as indicated. Samples (500 μL) were taken according to a suitable time schedule (0.1 - 1 h for initial rates), heat-inactivated and analyzed for the Glc-1-P synthesized.

Pretreatment of α-glucans by pullulanase (60-240 PUN·g^{-1} glucan) was carried out (i) at 50 °C in 50 mM citrate buffer (pH 5.0) or (ii) at the particular conditions for the simultaneous action with phosphorylase. The time course of substrate pretreatment by pullulanase was semi-quantitively determined by the formation of iodine-starch complexes as recently reported[19] or by the release of reducing sugars measured with the dinitrosalicylic acid reagent.[25]

Continuous experiments were carried out at 30 °C in a convective, well mixed, thermostated enzyme reactor with a total volume of 40 - 70 mL. The reactor was equipped with a flat, 30 kDa cut-off ultrafiltration membrane (Millipore) completely retaining the phosphorylase, a sensor to monitor the pressure inside the reaction system and a magnetic stirrer (3-6 s^{-1}). Substrate (pretreated or non-treated glucan and phosphate) was fed through a sterile filter and the average residence time in the reactor was varied as indicated. The reaction was started by the injection of a concentrated phosphorylase preparation (30-50 $U \cdot mL^{-1}$) through a septic seal to give an approximate volumetric phosphorylase activity of 1 $U \cdot mL^{-1}$. Samples were then periodically taken at the reactor outlet and assayed for Glc-1-P concentration. For activity measurements samples were taken from the reactor, gel-filtered using NAP5 columns (Pharmacia) to remove Glc-1-P and substrates, and assayed for phosphorylase activity as described above.

Computational. Kinetic constants were calculated by non linear regression analysis employing the BMDP statistical software package (BMDP).

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