Acarbose 7-Phosphotransferase from *Actinoplanes* sp.: Purification, Properties, and Possible Physiological Function

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A phosphotransferase which modifies the α-glucosidase inhibitor acarbose by phosphorylation at its 7-position was isolated from the acarbose producer *Actinoplanes* sp. and purified to homogeneity. The sequence of the first 20 amino acids of the enzyme was determined. The enzyme is an ATP-dependent kinase and shows high specificity for acarbose and some related compounds containing the pseudodisaccharide moiety (acarviosin). The product formed by the enzyme, acarbose-7-phosphate, shows a significant lower inhibitory activity towards disaccharidases than acarbose itself. The acarbose producing organism contains a maltase which is inhibited by acarbose, but to a much lesser extent by acarbose-7-phosphate. The possible role of acarbose 7-phosphotransferase as part of a self-defense mechanism against acarbose in the producing organism is discussed.

As described in the previous paper[1], the α-glucosidase inhibitor acarbose is transformed to its 7-phosphate by a cell-free extract from the acarbose-producing organism *Actinoplanes* sp. This modification reaction bears some resemblance to phosphorylations in the aminoglycoside antibiotic series catalyzed by aminoglycoside phosphotransferases[2]. These enzymes generally appear to confer resistance against aminoglycoside antibiotics both in aminoglycoside-producing and non-producing bacteria. Comparing their chemical structures and modes of action, acarbose and aminoglycoside antibiotics differ quite significantly: the former can be viewed as a pseudotetrasaccharide analogue of maltotetraose and is a highly specific inhibitor of α-glucosidases[3], the latter usually contain a diaminocyclitol glycosidically linked to a wide variety of unusual sugars and inhibit bacterial protein synthesis by interference with ribosomal function[4]. The non-reducing end of acarbose is the unsaturated "aminocyclitol"-moiety valienamine which is also found in the validamycin antibiotics[5,6]. To our knowledge, the formation of acarbose phosphate is the only reported modification by phosphorylation in this series of secondary metabolites. This paper describes the enzyme catalyzing the ATP-dependent phosphorylation of acarbose and some properties of its product which may be related to the physiological role of the enzyme.

Materials and Methods

Culture Conditions

Actinoplanes sp. SN 223/29 was cultivated in a two stage complex medium. Preculture: soy flour (Hensel-werk, Magstadt, Germany), extracted with aceton 2%; glycerol 2%; $CaCO_3$ 0.2%; tap water. pH was adjusted to 7.2 before sterilisation. The medium was inoculated with 4.5 ml of 72 hours old preculture stored at −20°C. Main culture: maltzin (Diamalt, Munich) 7.5%; STV yeast extract (Ohly, Hamburg) 0.7%; NZ amine (Sigma) 0.3%; $CaCO_3$ 0.3%; K_2HPO_4 0.3%; tap water. Inoculum was 9 ml of 72 hours old preculture. Both cultures were grown in 1 liter Erlenmeyer flasks with 125 ml medium at 28 °C with 260 rpm on a rotary shaker.

Determination of Acarbose by Saccharase Inhibition Assay

The procedure of MÜLLER *et al.*[6] was used.

Determination of Enzyme Activities

Cells were harvested by centrifugation. Pellets were washed three times and stored at −20°C. Thawed cells (2 g) were suspended in buffer (3 ml 25 mM Tris-HCl, 25 mM $MgCl_2$, 63 mM NH_4Cl (pH 7.8), 1 mM mercaptoethanol) and disrupted by sonication (Branson Sonifier W-250: step 5 ~ 7, 25%, 8 ~ 30 minutes depending on the volume). Debris was separated from soluble substances by centrifugation (30000 *g*, 4°C, 60 minutes). Supernatants were used in enzyme assays.

Acarbose kinase activity was measured photometrically in an assay coupled with pyruvate kinase (220 nkat/ml) and lactate dehydrogenase (820 nkat/ml) in 65 mM Tris-HCl buffer (pH 7.8), 3.3 mM $MgCl_2$, 1.1 mM ATP, 1.6 mM PEP (potassium salt) and 0.5 mM NADH. The reaction was started with acarbose (6.6 mM) or other substrates (Table 2). The oxidation of NADH was registered at 340 nm. Protein concentration was measured according to BRADFORD[7].

Maltase activity: 500 μl test reagent (containing each 10 U/ml glucose oxidase and peroxidase, 2 mM ABTS

[2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid)] in 0.2 M sodium phosphate buffer, pH 7.2), 375 µl A. demin., 100 µl desalted crude extract (0.1 to 0.6 mg/ml protein) and 25 µl 0.72 M maltose were mixed and preincubated for 3 minutes at 30°C. Oxidation of ABTS was continuously recorded for further 5 minutes. Blanks without the addition of crude extract were used to correct for glucose contamination of maltose. Different inhibitor concentrations (acarbose, acarbose-phosphate) were added in 50 µl of A. demin.

Purification and Sequencing of Acarbose Kinase

Acarbose kinase was purified in a 6 step protocol. Step 1: crude extract from 60 g 120 hours old cells (main culture) was prepared as described above. Step 2: 100 ml extract were desalted by gel filtration with Sephadex G-25. The column was washed with the same buffer as used for sonication. Step 3: 50 ml 0.5 M sodium acetate pH 4.75 were slowly added to 140 ml desalted crude extract and precipitated proteins were separated by centrifugation (20 minutes, 30000 *g*). The supernatant was adjusted to pH 6.0 by titration with 1 M Tris and concentrated by ultrafiltration (PM 30, Amicon) to a volume of 100 ml. Buffer was changed by a further gel filtration with Sephadex G-25 (50 mM Tris-HCl, 5 mM $MgCl_2$ pH 7.5). Step 4: chromatography on Q-Sepharose FF; elution followed with a gradient from 0 to 1 M NaCl. Active fractions were brought to 1.2 M $(NH_4)_2SO_4$ for stabilisation of enzymatic activity. Step 5: chromatography on Alkyl-Superose HR 5/5 (FPLC) using the same buffer; elution followed with a gradient from 1.2 to 0 M $(NH_4)_2SO_4$. Pooled active fractions were dialyzed against 0.1 M NH_4HCO_3, freeze dried and stored at −20°C. Step 6: preparative SDS-PAGE[8] with 10.7% (w/v) acrylamid and 0.3% (w/v) bisacrylamid in 375 mM Tris buffer pH 8.8. Electrode buffer: 50 mM trizma, 0.4 M glycin, 0.1% (w/v) SDS, (pH 8.5). Cathode buffer contained 0.1 mM thioglycolic acid.

After electrophoretic separation glycine was removed by washing the gel 3 × 20 minutes in bidestillated water. Then proteins were blotted on PVDF membrane (transfer buffer: 13 mM Na_2CO_3 pH 9.9 with 20% methanol). The blot was stained with Coomassie brilliant blue R250. The *N*-terminus was sequenced (after pyridylethylation[9]) with an Applied Biosystems protein sequencer.

Determination of Molecular Weight

Acarbose kinase was partially purified by the described procedure (step 1 to 3) and ion exchange chromatography (DEAE-Sephacel; buffer: 10 mM Tris-HCl pH 7.5, 1 mM β-mercaptoethanol, elution with 0 to 1 M NaCl). The molecular weight was determined by molecular sieve chromatography on Sephacryl S-300 (column: 97 × 1.5 cm; buffer: 50 mM Tris-HCl pH 7.5, 1 mM β-mercaptoethanol).

Purification of Acarbose-7-phosphate

The reaction mixture for enzymatic synthesis of acarbose phosphate contained 20 ml desalted crude extract, 200 mM Tris-HCl pH 7.7, 50 mM $(NH_4)_2SO_4$, 15 mM ATP (pH 7.7), 10 mM acarbose and 5 mM $MgCl_2$ in a total volume of 50 ml. After incubation for 2 hours at 37°C the reaction was stopped by adding 50 ml ethanol and precipitated protein was separated by centrifugation (20 minutes, 30000 *g*). The supernatant was freeze dried.

The crude product was purified by chromatography on QAE-Sephadex A-25. The sample (0.82 g) was dissolved in 200 ml 2 mM NH_4HCO_3 (pH was adjusted to 10.0 with NH_3) and applied to the column. Elution followed with a gradient from 2 mM to 0.5 M NH_4HCO_3 (pH 10.0).

Acarbose phosphate concentration was determined in the following way: the phosphate ester was hydrolysed with alkaline phosphatase (20 mM acarbose phospate, 150 mM diethanolamine pH 9.0, 1 mM $MgCl_2$, 500 U/ml alkaline phosphatase (Boehringer, Mannheim); incubation for 30 minutes at 37°C), and the resulting products phosphate and acarbose were quantitated by phosphate assay[10] and saccharase inhibition assay[6].

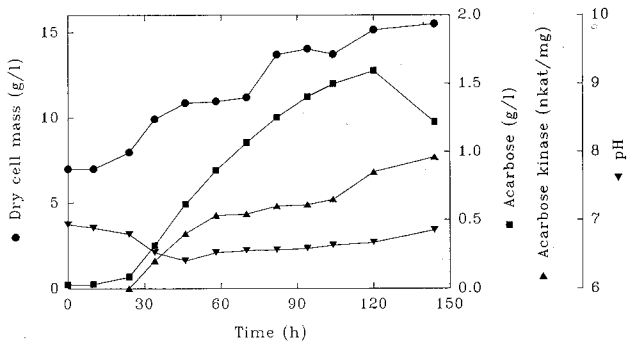
Acarbose (BAY-g-5421), components 2, 4b, A, C, BAY-o-4975, JUB 1026-6-8, 2 C_7-cyclites und validamycin (techn.) and disaccharidase mixture from pig small intestine mucosa were gifts from Bayer AG, Leverkusen, Germany.

Results and Discussion

Acarbose kinase is located in the cytoplasm and is produced roughly parallel to the synthesis of acarbose as shown in Fig. 1. To get more information on this interesting enzyme we attempted the purification and characterization of the enzyme.

Mycelia of the acarbose producing organism were grown in a complex medium—where both acarbose and acarbose kinase activity are produced—and harvested after 120 hours. Cells were broken by sonication and the acarbose kinase was purified by a 5 step procedure

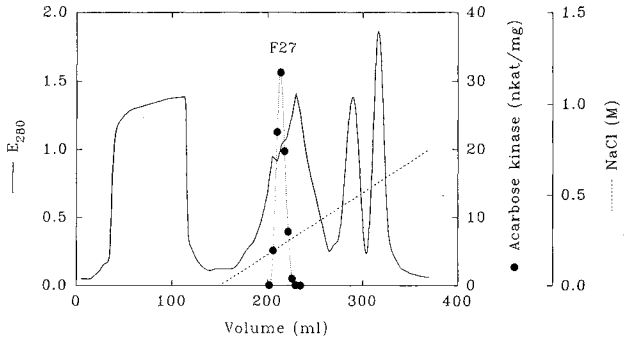
Fig. 1. Growth, acarbose production and activity of acarbose kinase during submerged fermentation of the acarbose producer *Actinoplanes* sp. SN 223/29 in complex medium.



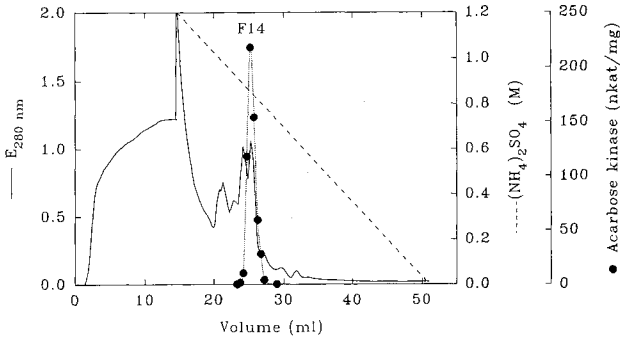
involving gel filtration, acid precipitation, ion exchange chromatography (Fig. 2A) and hydrophobic chromatography (Fig. 2B). Approximately 89-fold enrichment was achieved in this way (Table 1).

Fig. 2. Purification of acarbose kinase.

A) Chromatography on Q-Sepharose FF: the supernatant (188 mg protein) after step 3 was applied to the column and eluted with a gradient from 0 to 1 M NaCl; fractions 25 to 29 (41 mg protein) were pooled.



B) Chromatography on Alkyl-Superose HR 5/5 (FPLC): pooled active fractions from the IEC were brought to 1.2 M $(NH_4)_2SO_4$ and applied to the column; protein was eluted with a gradient from 1.2 M to 0 M $(NH_4)_2SO_4$; fractions with acarbose kinase activity were pooled (3.2 mg protein).



Properties of Acarbose Kinase

Molecular weight of the enzyme was found to be 36.2 kDa by molecular sieve chromatography (data not shown). This corresponds with the results of gel electrophoresis of the purest fractions (step 5): the preparation showed one main band representing a protein with a size of approximately 36 kDa and some faintly stained contaminating bands (Fig. 3).

Kinetic properties were determined with an enzyme preparation purified approximately 10 fold by ion exchange chromatography (Table 1, step 4). The apparent Michaelis constants for acarbose and ATP were 0.81 mM and 0.47 mM, respectively. Compared with the majority of aminocyclitol phosphotransferases for a wide range of bacteria these values appear to be rather high[11].

Fig. 3. SDS-PAGE after different steps of the purification of acarbose kinase.

lane 1 and 7: marker proteins, lane 2: crude extract (5 μg) (step 1), lane 3: desalted crude extract (5 μg) (step 2), lane 4: desalted supernatant after precipitation with NaAc-buffer (5 μg; step 3.d), lane 5: fractions 25 to 29 from chromatography on Q-Sepharose (2.5 μg; step 4), lane 6: pooled fractions after chromatography on Alkyl-Superose and dialysis (1.5 μg; step 5).

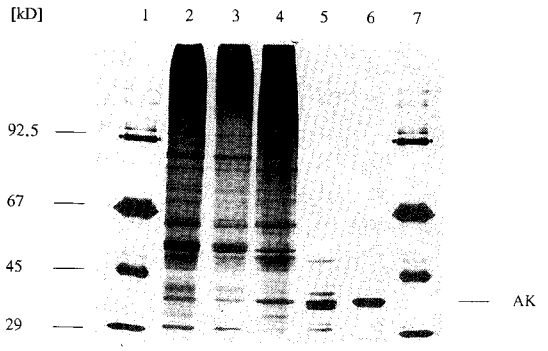


Table 1. Overview of the purification of acarbose kinase from crude extract of the acarbose producer *Actinoplanes* sp. SN 223/29.

Purification step	Specific activity (nkat/mg)	Total activity (nkat)	Recovery (%)	Purification factor
1. Crude extract	1.79	819	100	1.0
2. Desalted crude extract	2.00	1162	142	1.1
3. Precipitation with sodium acetate-buffer pH 4.75:				
a) Precipitate	0.02	4.2	0.5	0.0
b) Supernatant	4.20	784	96	2.4
c) Concentrated supernatant	4.69	882	108	2.6
d) Concentrated supernatant after desalting 4.37	820	100	2.4	
4. IEC on Q-Sepharose:				
Fraction 25 to 29	17.3	762	93	9.7
5. Hydrophobic chromatography on Alkyl-Superose:				
Pooled fractions from 2 runs	159	516	63	88.8

Substitution of acarbose in the kinase assay by a series of acarbose related compounds, sugars and aminocyclitol antibiotics revealed a high specificity for the pseudodisaccharidyl unit (Table 2). Aminocyclitol antibiotics without a valienamine moiety are not phosphorylated. Validamycine which contains valienamine but no pseudodisaccharide, a valienamine free homolog of acarbose and two synthetic branched chain C7-cyclitols are not significantly phosphorylated.

The nature of the sugar chain attached to the pseudodisaccharide exhibits a significant influence on the phosphoryl acceptor activity. Several maltose homologs containing maltotriose or modified disaccharides instead of maltose are less well phosphorylated than acarbose (caused by limited availability of acarbose homologs with saccharose, trehalose of maltotriose units instead of maltose, these measurements were done at lower substrate concentrations. However, the applied concentrations were within saturation range of acarbose for the kinase, according to the kinetic data). Only pseudodisaccharidyl-glucose and the methylglycoside of acarviosin are similar effective substrates as acarbose. Glucose and glucooligosaccharides including the acarbose-analogue maltotetraose are not phosphorylated by acarbose kinase.

Table 2. Phosphorylation of different substrates by acarbose kinase.

Substrate	Concentration in the test (mM)	Activity (%)
Acarbose*	10.0	100
Component 2*	10.0	110
Component 4b*	1.3	10.7
Component A*	5.0	49.5
Component C*	5.0	22.0
Bay-o-4975*	10.0	96.7
JUB 1026-6-8*	10.0	1.2
C7-cyclitol (A)*	10.0	6.0
C7-cyclitol (B)*	10.0	1.0
Glucose	7.5	0
Maltose	7.5	0
Maltotriose	6.6	0
Maltotetraose	6.6	0
Streptomycin	10.0	0
Kanamycin	10.0	0
Neomycin	10.0	0
Validamycin	10.0	3.0

Activity with acarbose was set as 100%. Purified acarbose kinase (step 4 as presented in Table 1) was used.

* The structure of these substances are given in Fig. 4.

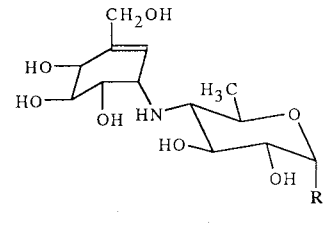
N-terminal Sequence of Acarbose Kinase

The 36 kDa protein was isolated after preparative SDS-PAGE and subjected to Edman degradation for the determination of the *N*-terminal amino acid sequence. The data indicate the following *N*-terminal sequence:

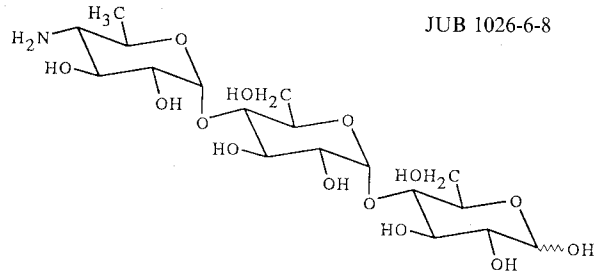
1	2	3	4	5	6	7	8	9	10	
ser	glu	his	thr	asp	val	leu	val	leu	gly	
11	12	13	14	15	16	17	18	19	20	...
gly	ala	gly	val	asp	X	ile	ala	tyr	val	...

Except position 16 all amino acids up to position 20 could be unequivocally identified. The *N*-terminus starts with serine instead of methionine. A search for homologies showed no similarity with known proteins (protein data bank Swiss Prot, DKFZ Heidelberg).

Fig. 4. Structures of compounds tested for phosphorylation by acarbose kinase.

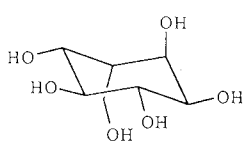


	R
Acarbose:	-(4-O) maltose
Component 2:	-(4-O) glucose
Component 4b:	-(4-O) maltotriose
Component A:	-(4-O) saccharose
Component C:	-(4-O) trehalose
Bay-o-4975:	-methyl



JUB 1026-6-8

Cyclitol (A)



Cyclitol (B)

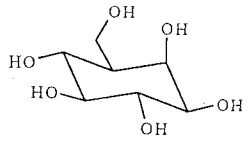


Fig. 5. Inhibition of disaccharidase (from mucosa of pig small intestine) by acarbose and acarbose-7-phosphate (saccharase inhibition assay).

● Acarbose, ▼ acarbose-7-phosphate.

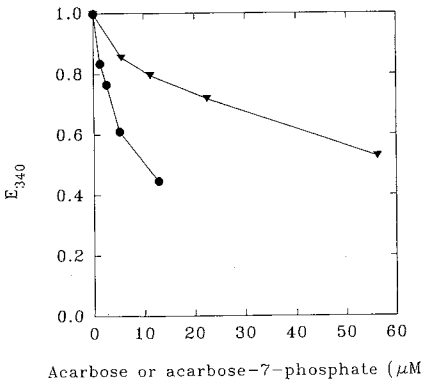
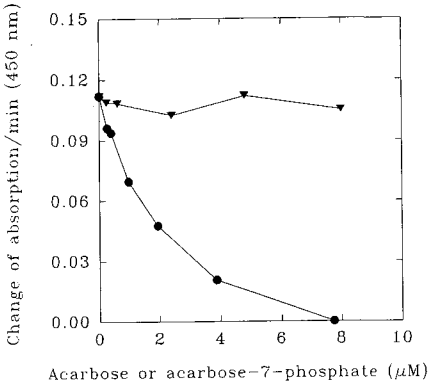


Fig. 6. Inhibitory influence of acarbose and acarbose-7-phosphate on the activity of maltase from the acarbose producer *Actinoplanes* sp. SN 223/29.

● Acarbose, ▼ acarbose-7-phosphate.



Crude extract from 50 hours old cells was desalted (Sephadex G-25) and used as described.

Possible Physiological Function of Acarbose Kinase

Among actinomycetes the occurrence of aminocyclitol phosphotransferases is usually coupled with the ability to synthesize these antibiotics. This phenomenon is generally regarded as part of a self defense mechanism, since the antibiotic activity of the amino cyclitols is diminished by phosphorylation.

We therefore compared the inhibitory activities of acarbose and its 7-phosphate in the standard disaccharidase inhibition assay. Acarbose-phosphate was synthesized enzymatically from acarbose and ATP and purified as described above. As shown in Fig. 5, a disaccaridase mixture from pig small intestine mucosa using saccharose as substrate is much less sensitive to acarbose-7-phosphate compared to acarbose.

Maltose and maltooligosaccharides are the predominant carbon substrates during fermentation of the acarbose producing Actinoplanes. Under these conditions significant amounts of acarbose are produced (Fig. 1). The organism contains a cytoplasmic α-glucosidase activity (maltase) probably involved in utilisation of the substrates. As shown in Fig. 6, this maltase activity is severely inhibited by acarbose but not by acarbose-7-phosphate. This finding indicates that acarbose kinase may perhaps be necessary for the organism to allow substrate utilisation during the synthesis of acarbose.

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