Processes for preparing acarviosyl transferase and for using it in the conversion of acarbose homologues into acarbose, for the preparation of acarbose homologues

The invention relates to acarviosyl transferase (AT) from actinomycetes, mainly from Actinoplanes sp. SE 50/13 or SE 50/110 and its mutants, to a process for isolating, purifying and characterizing the enzyme, to the isolation and characterization of the acbD gene encoding the acarviosyl transferase, to the expression of acarviosyl transferase in a heterologous host organism, to the use of acarviosyl transferase for converting acarbose minor constituents into acarbose or for preparing acarbose homologues, to the use of acarviosyl transferase in acarbose purification and to the preparation of production mutants in which formation of minor constituents is reduced by means of inactivating the acarviosyl transferase.

The discovery that a series of actinomycetes, especially the Actinoplanaceae, form oligosaccharide-like inhibitors of glycoside hydrolases, primarily carbohydrate-cleaving enzymes of the digestive tract, is the subject-matter of previous patent applications (e.g. DE 20 64 092 and DE 22 09 834). The inhibitors consist of an acarviosyl unit which is linked α-1,4-glycosidically to maltooligosaccharides or other sugars. The acarviosyl core can be linked on both sides to differing numbers of glucose units. The number of glucose residues on the core determines the specific activity of the inhibitor. Relatively short molecules (components containing 1-5 glucose units) act mainly on disaccharidases, whereas the effect on α-amylases becomes more efficient as the number of glucopyranoses increases. As the most potent α-glucosidase inhibitor of this group, the compound O-4,6-dideoxy-4-[[1S-(1S,4R,5S,6S)-4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]-amino]-D-glucopyranosyl-(14)-O-D-glycopyranosyl-(14)-D-glucopyranose is described as acarbose [DE 23 47 782].

Acarbose is employed in human medicine in the control of diabetes mellitus. The secondary metabolite acarbose is formed by Actinoplanes sp. SE 50 (CBS No. 961.70)

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and by a natural variant of this strain, SE 50/110 (CBS 674.73), or else by SE 50/13 (CBS 614.71) [DE 22 09 83] and by their selectants and mutants. The isolation of a saccharase inhibitor of this nature is described in the patent applications mentioned, for example in Examples 1 to 4 of the said German Patent Application P 20 09 834. In association with this isolation, acarviosyl-containing compounds possessing differing numbers of maltooligosaccharide and disaccharide residues appear as minor constituents in addition to acarbose as the main product.

Acarbose consists of an acarviosyl residue which, according to the current state of knowledge, is formed first during the biosynthesis in order then to be linked to a maltosyl residue. In 1986, during the course of studies designed to elucidate the biosynthesis of acarbose, Goeke (Goeke, K., Enzymatische Untersuchungen zum Zuckerstoffwechsel und zur Biosynthese des α-Glucosidase-Inhibitors Acarbose bei Actinoplanes spec. [Enzymic investigations on sugar metabolism and on the biosynthesis of the α-glucosidase inhibitor acarbose in Actinoplanes spec.]; Dissertation, Münster University) described an enzymic exchange of the maltosyl residue of the acarbose (acarviosyl-maltose) for a radioactively labelled maltose: when [U-^{14}C]-maltose is used, an acarbose is formed which is radioactively labelled in the maltosyl residue. The enzyme involved, i.e. acarviosyl transferase (AT), was initially termed pseudodisaccharidyl(PDS) transferase. From the fact that the activity of the pellet fraction, following cell disruption and differential centrifugation, was greater by a factor of 3.25 than the acarbose/maltose exchange reaction of the supernatant fraction, it was concluded that the PDS transferase was membrane-bound. In 1991, Schaper (Schaper, B., Biochemische und physiologische Studien zur Biosynthese des α-Glucosidase-Inhibitors Acarbose [Biochemical and physiological studies on the biosynthesis of the α-glucosidase inhibitor acarbose]; Dissertation, Münster University) confirmed this finding and elaborated a detailed working-up procedure for the membrane-bound enzyme. A pH optimum of 4.5 was cited for the partially purified enzyme, at a temperature optimum of 30°C and using Mn^{2+} as a cofactor.

It has now been found, surprisingly, that the acarviosyl transferase which possesses the ability to exchange the maltosyl residue of acarbose is not present in membrane-bound

form; on the contrary, the AT is mainly present in the culture filtrate, with the enzyme activity increasing in parallel with cell growth. It was possible to isolate the enzyme at high purity from the cell supernatant. For this purpose, the enzyme was precipitated from the culture supernatant using ammonium sulphate. After centrifugation, the sediment was dissolved in buffer (containing glycerol and $CaCl_2$), after which this solution was centrifuged once again and the resulting supernatant was passed through an anion exchange column. The run-through contained the acarviosyl transferase. The AT was obtained from this solution by dialysis as a partially enriched preparation, or else purified by chromatography on DEAE-Fractogel®, precipitating twice with sedimentable starch and desorbing with acarbose or maltose.

The purified acarviosyl transferase has a MW of 76 kDa (SDS-PAGE) and a temperature optimum of 20-40°C. The enzyme is temperature-stable up to approx. 40°C. A pH optimum of 6.2-6.9 was determined, as was a dependency on Ca^{2+} ions.

The purified enzyme was sequenced. Surprisingly, the resulting base sequence exhibits good agreement with the corresponding DNA sequence of the acb*D* gene from the acarbose gene cluster of the producing organism Actinoplanes sp. SE 50/110.

It is furthermore surprising that acarviosyl transferase can replace the maltosyl residue of acarbose with other sugar residues, with the formation of acarbose homologues, or is able to form acarbose by replacing the sugar residues of the acarbose-like minor constituents, which are formed during the fermentation, with a maltose residue. In this context, acarviosyl transferase is catalyzing the general reaction

acarviosyl-X + Y → acarviosyl-Y + X

(X = glucose, maltose, maltooligosaccharides and other sugars,

Y = glucose, maltose, maltooligosaccharides and other sugars).

The invention therefore discloses:

The complete process for isolating and purifying acarviosyl transferase from cultures of Actinoplanes sp. SE 50/110 or its mutants.

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The characterization of the purified acarviosyl transferase.

The amino acid sequence which was determined over more than 100 amino acids following tryptic cleavage of the enzyme. The base sequence which is obtained from this amino acid sequence exhibits good agreement with the base sequence of the acbD gene.

A process for preparing acarbose from acarbose minor constituents by replacing the respective sugar residues with maltose.

A process for preparing acarbose homologues possessing altered pharmacological properties by means of replacing the maltosyl residue of acarbose with other suitable sugar residues.

The use of acarviosyl transferase, or immobilized AT, for isolating acarbose from the culture broth (affinity chromatography) while at the same time converting acarbose homologues into acarbose.

The isolation and characterization of the acbD gene encoding acarviosyl transferase.

The recombinant preparation of acarviosyl transferase in an heterologous host organism.

The preparation of improved production mutants, with the product spectrum in Actinoplanes being restricted to acarbose, as the desired main product of the biosynthesis, by means of switching off the unwanted formation of minor constituents by inactivating the acbD gene.

The invention is described in detail below. The invention is furthermore determined by the content of the claims.

The invention is described in detail below.

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I. Enzyme purification

Culture filtrate from the two-phase growth of *Actinoplanes* sp. SE 50/110 or its mutants:

Seed culture:	soya bean meal, defatted	2%
	glycerol	2%
	$CaCO_3$	0.2%
	tap water	
	pH 7.2, adjusted using NaOH	
	1000 ml Erlenmeyer flask, filled to a volume of 125 ml	
	inoculum: 5 ml of stock culture (72h seed culture, storage at 20°C)	
	incubation: 72 h at 30°C and 260 rpm	
Main culture:	soya bean meal, defatted	1%
	starch	3%
	$CaCO_3$	0.2%
	tap water	
	1000 ml Erlenmeyer flask, filled to a volume of 125 ml	
	inoculum: 5 ml of seed culture	
	incubation: 96-144 h at 30°C and 260 rpm.	

The maximum AT activity, of 2.6 nkat/ml of culture filtrate, is reached after a culturing period of 120 h. AT is then the quantitatively predominant protein in the culture filtrate.

The purification scheme is depicted in Table 1.

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Table 1 Scheme for purifying acarviosyl transferase

The following buffers were used:

Buffer 1: 25 mM Tris/HCl pH 8.5 + 10% glycerol + 1 mM $CaCl_2$

Buffer 2: 25 mM Tris/HCl pH 7.5 + 1 mM $CaCl_2$

Buffer 3: 10 mM Tris/HCl pH 7.5 + 1 mM $CaCl_2$

Buffer 4: 0.1 mM Tris/HCl pH 7.2 + 0.01 mM $CaCl_2$

Starch*: boiled, soluble starch, 12 h at 4°C ("cold precipitation"), centrifugation at 40,000 × g for 60 min; the sediment is employed.

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Soya bean meal/starch culture

↓ 3,000 × g; 10 min

Culture filtrate

| fractional $(NH_4)_2SO_4$ precipitation (20-40% saturation)

↓ 25,000 × g; 30 min (2 ×)

Sediment

| solubilization in Buffer 1

↓ 25,000 × g; 30 min

Supernatant

↓ DEAE-anion exchange column; 0-1 M NaCl

Run-through

↓ dialysis (12 h), Buffer 1

Retentate

↓ DEAE-anion exchange column; 0-1 M NaCl

0.15-0.35 M NaCl fractions

| incubation with starch* (12 h)

↓ 40,000 × g; 60 min

Sediment (in Buffer 2)

| incubation with acarbose (25 mM; 2 h at RT)

↓ 40,000 × g; 60 min

Supernatant

| dialysis (3 × 6 h) using Buffer 3

↓ incubation with starch* (12 h); 40,000 × g; 60 min

Sediment (in Buffer 3)

| incubation with maltose (250 mM; 12 h)

↓ 40,000 × g; 60 min

Supernatant

↓ dialysis (3 × 6 h) using Buffer 4

Purified AT

The purification is summarized in Table 2

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Table 2: Purification of acarviosyl transferase, summary (for the sample designation, see the purification scheme in Tab. 1)

Purification step	Total protein (mg)	Total activity (nkat)	spec. activity (nkat/mg)	Yield (%)	Enrich-ment
Culture filtrate	400	1740	4.35	100	1
20-40% $(NH_4)_2SO_4$	139	1147	7.75	66	1.8
Run-through 1st column	89	1078	12.1	62	2.8
Active fraction 2nd column	20	789	38.8 (43.1)*	45	8.9
Starch precipitation + acarbose desorption	9.7	538	55.5	31	12.8
Starch precipitation + maltose desorption	5.5	425	77.3	24	17.8

* most active fraction

The purification of the AT results in a 17.8-fold enrichment with a yield of 24%.

II. Determination of acarviosyl transferase activity

1. Radioactivity test

a) Acarbose or acarbose homologues were incubated at 30°C with [^{14}C]-maltose in a tris-maleate buffer (pH 6.3) in the presence of preparations containing acarviosyl transferase. The acarbose was then separated from the maltose using a cation exchange resin. The level of [^{14}C] radioactivity in the acarbose fraction in relation to the total radioactivity gives the replacement rate, which is correlated with the AT activity.

Acarviosyl-(α-1,4)-(sugar) + [^{14}C]-maltose → acarviosyl-(α-1,4)-[^{14}C]-maltose + sugar

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b) [^{14}C]-Acarbose (labelled in the maltose unit) was incubated together with maltooligosaccharides or other sugars in the presence of AT, as in a). The mixture was treated and evaluated as described in a).
Acarviosyl-(α-1,4)-[^{14}C]-maltose + sugar → acarviosyl-sugar + [^{14}C]-maltose

2. Thin layer chromatography
Acarbose or acarbose homologues were incubated with maltose, maltooligosaccharides or other sugars, as described in a). The reaction mixture contained:
10 µl of AT preparation (AT in 0.1 mM Tris/HCl pH 7.2 + 0.01 mM $CaCl_2$; 4.5 nkat/ml)
10 µl of acarbose (70 mM stock solution) or acarbose homologues (approx. 30 mM)
10 µl of substrate (70 mM stock solution) or maltose (600 mM)

Sample preparation

30 µl of reaction mixture
↓ 18 h; 30°C
addition of 70 µl of ethanol
↓ centrifugation for 5 min at 7000 g
removal of 80 µl of supernatant → 5 µl for the TLC
↓
75 µl
↓ drying in a vacuum concentrator
sample for HPLC

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TLC: silica gel 60 TLC aluminium foils (Merck), mobile phase: butanol: ethanol: water (50:30:20); staining: Cer spray reagent; development at 110°C.

3. HPLC Test

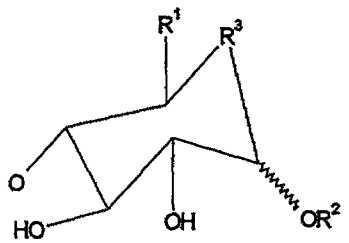
Acarbose or acarbose homologues were incubated with maltose, maltooligosaccharides

or other sugars as described in 2. After precipitating the proteins, the chemical composition of the remaining solution was analyzed by means of ECD HPLC or UV HPLC.

III. Properties of acarviosyl transferase

Molar mass	76 kDa (SDS-PAGE)
pH optimum	6.2-6.9
Temperature optimum	30°C (20-40°C)
Temperature stability	up to approx. 40°C
Trace element dependence	Ca^{2+}

The following general formula for the acceptor molecule can be deduced on the basis of the acceptor speficities which are listed below.



R^1 is H, CH_2OH or CH_3

R^2 is H, $(CH_2)_mCH_3$, m = 0-10

pyranoses [α(1->2), (1->3), (1->4), (1->6), β(1->2), (1->3), (1->4)]

furanoses [α(1->6)]

glucitol, phenyl-, nitrophenyl-, etc.

R^3 is O, S or CHOH

Acceptor specificity

cellobiose

deoxy-D-glucose

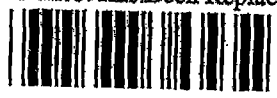
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D-gluconic acid lactone
D-glucose
isomaltose
isomaltotriose
laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucose)
maltose
maltotriose
maltotetraose
maltopentaose
maltohexaose
maltoheptaose
methyl-D-glucopyranoside
palatinose
panose (6-α-glycosyl-maltose)
sophorose (2-*O*-β-D-glucopyranosyl-α-D-glucose)
xylobiose
L-xylose
D-xylose
nigerose
L(-)-glucose
5-thio-D-glucose
myo-inositol
maltitol
amygdalin
amylopectin
dextrin
α-D(+)-maltose-1-phosphate
4-nitrophenyl-α-D-glucopyranoside
4-nitrophenyl-β-D-xylopyranoside
D(-)-salicin
phenyl-α-D-glucopyranoside
octyl-D-glucopyranoside

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	nonyl-β-D-glucopyranoside
	octyl-β-D-maltopyranoside
	decyl-β-D-maltopyranoside
Donor specificity	acarbose
	acarbose minor constituent 2
	acarbose minor constituent 4A
	acarbose minor constituent 4B
	acarbose minor constituent 4C
	acarbose minor constituent B
	pseudoacarbose

IV. Protein sequencing

The N-terminal sequences of fragments of acarviosyl transferase were analyzed using an Applied Biosystems 473A gas-liquid solid-phase protein sequencer (Foster City, CA., U.S.A.). The standard sequencing programme for this equipment was used. The appliance, the programmes which are used and the PTH separation system are described in detail in the user's manual (user's manual protein sequencing system model 473A (1989), Applied Biosystems, Foster City, CA 94404, U.S.A.).

The PTH amino acids were detected on-line using an Applied Biosystems RP-18 column (220 mm × 2 mm, 5µ material). Identification and quantitative determination of the PTH amino acids were effected with the aid of a 50 pM standard of all the PTH amino acids. The Applied Biosystems Sequencer Data System 610A was used for evaluating the data. The chemicals which were used for the protein sequencer were obtained from Applied Biosystems.

The Pharmacia (Freiburg, Germany) Smart System was employed for separating the tryptic peptides.

The HPLC column (2.1 mm × 100 mm; 5µ material) for separating the tryptic peptides

was obtained from Pharmacia (Freiburg, Germany), while trypsin (sequence grade) was obtained from Boehringer Mannheim and all the remaining chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany).

V. Isolation and sequencing of the acbD gene (AcbD protein = AT)

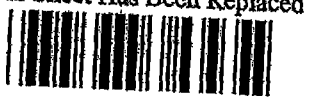
Unless otherwise indicated, all the genetic manipulation methods were carried out as described in Sambrook et al. (Molecular Cloning; A laboratory manual; 2nd edition, 1989; Cold Spring Harbour Laboratory Press, N.Y., U.S.A.).

The gene probe which was used for the screening was isolated from the plasmid pAS2 (DE 195 07 214). Plasmid pAS2 was prepared from E. coli DH5α by means of the boiling method or by alkaline lysis and hydrolyzed with the restriction endonuclease BamHI. The resulting 2.2 kb BamHI fragment was isolated and radioactively labelled with ^{32}P-labelled deoxynucleotides by means of nick translation. This labelled fragment was employed as a gene probe for isolating acarbose biosynthesis genes (DE 195 07 214) and is termed acb probe II in that which follows.

Acarbose biosynthesis genes were isolated as follows. Chromosomal DNA from Actinoplanes sp. SE 50/110 was hydrolyzed with the restriction enzyme SstI, separated by gel chromatography and examined by means of Southern Hybridization, using the acb probe II, for the presence of an homologous DNA sequence. The SstI fragment which was hybridized by the gene probe had a size of 11 kb. The 11 kb SstI fragment was eluted from the gel and ligated into the vector pUC18; this recombinant vector was then cloned into E. coli DH5α. The resulting plasmid was given the designation pAS5. Plasmid pAS5 was hydrolyzed with the restriction enzymes PstI and HindIII. The resulting fragments had the following sizes:

1.4 kb PstI fragment
5.4 kb PstI fragment
0.05 kb PstI/HindIII fragment
2.6 kb HindIII/PstI fragment

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3.8 kb PstI fragment (1.1 kb PstI/SstI fragment ligated to the pUC18 vector)

The 2.6 kb HindIII/PstI fragment was eluted from the gel and ligated into the pUC18 vector; this recombinant vector was then cloned in E. coli DH5α. The resulting plasmid (pAS5/15.1) was hydrolyzed with various restriction endonucleases and the resulting DNA fragments in pUC18 were subcloned in E. coli DH5α and sequenced. In order to check the DNA sequences of the fragments derived from pAS5/15.1, DNA fragments were also amplified from Actinoplanes sp. chromosomal DNA by the PCR method, ligated into pUC18, cloned in E. coli DH5α and then sequenced. The DNA sequences of the PCR primers were deduced from the DNA sequences of the subcloned fragments from pAS5/15.1 (see below).

For determining the DNA sequence of the 2.6 kb HindIII/PstI fragment from Actinoplanes sp., the following plasmids were constructed and the sequence of the inserted DNA was determined in each case:

pAS5/15.1	= 2.6 kb HindIII/PstI fragment from pAS5
pAS5/15.2	= 0.75 kb SalI fragment from pAS5/15.1
pAS5/15.3	= 0.5 kb SalI fragment from pAS5/15.1
pAS5/15.4	= 0.4 kb SalI fragment from pAS5/15.1
pAS5/15.5	= 0.35 kb SalI fragment from pAS5/15.1
pAS5/15.6	= 1.25 kb PvuII fragment from pAS5/15.1
pAS5/15.7	= 0.7 kb PvuII/HindIII fragment from pAS5/15.1
pAS5/15.9	= 0.1 kb PvuII fragment from pAS5/15.1
pAS5/15.12	= 0.9 kb KpnI/NcoI fragment from pAS5/15.1
pAS5/18	= 0.3 kb PCR fragment (primer: see Tab. 3)
pAS5/19	= 0.3 kb PCR fragment (primer: see Tab. 3)

The method of Sanger et al. (1977), or a method derived from this, was used for the DNA sequencing. The work was carried out using the Autoread Sequencing kit (Pharmacia, Freiburg, Germany) in combination with the Automated Laser Fluorescence (A.L.F.) DNA sequencing appliance (Pharmacia, Freiburg, Germany). Suitable fluorescein-labelled pUC reverse-sequencing and sequencing primers were obtained

commercially (Pharmacia, Freiburg, Germany).

Table 3 Sequences of the primers for the PCR and the sequencing reaction.

Primers for the PCR:

Plasmid pAS5/18:

Primer designation	Sequence
acbD3	5'ACCAGGCCGAGGACGGCGCCC3'
acbD4	5'AGCGGCATGTGCTTGACGGCG3'

Plasmid pAS5/19

Primer designation	Sequence
acbD5	5'ACCGGCTCGAACGGGCTGGCACC3'
acbD6	5'CCCTCGACGGTGACGGTGGCG3'

Primers for the sequencing reaction:

Primer designation	Sequence
universal primer	5'GTAAAACGACGGCCAGT3'
reverse primer	5'GAAACAGCTATGACCATG3'

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Examples

1. Preparation, purification and characterization of the acarviosyl transferase

After a seed culture on soya bean meal/glycerol medium, the wild-type strain Actinoplanes sp. 50/110, or a mutant derived from it, was fermented in the production culture on soya bean meal/starch medium at 30°C on an orbital shaker having a shaking frequency of 260 rpm. Following an incubation which lasted approx. 120 h, the cell mass was separated off. The enzyme was precipitated from the culture supernatant using ammonium sulphate (20-40% saturation). After centrifuging, the sediment was dissolved in buffer (25 mM tris/HCl, pH 8.5, containing a glycerol and $CaCl_2$) and this solution was centrifuged once again. The resulting supernatant was passed through a DEAE anion exchange column. The flow-through contained the acarviosyl transferase. From this solution, the AT was obtained as a partially enriched preparation by means of dialysis or was purified by means of chromatography on DEAE-Fractogel®, precipitating twice with starch and desorbing with acarbose or maltose (see Tab. 1). An enrichment by a factor of 17.8 was achieved in association with a 24% yield.

The activity of the acarviosyl transferase was measured by transferring the acarviosyl residue of acarbose (donor) to maltose (acceptor). The size of the enzyme was determined by SDS-PAGE to be 76,000 Da, with a pH optimum of 6.2-6.9 and a temperature optimum of between 20 and 40°C.

2. Sequencing the acarviosyl transferase

For determining the internal amino acid sequence, the acarviosyl transferase was digested with trypsin. Trypsin cleaves proteins after the amino acids lysine and arginine.

Tryptic cleavage of the AT: Approx. 1 mg of AT was dissolved in 1000 µl of 6M guanidinium chloride/0.5M tris-(hydroxymethyl)-aminomethane, pH 8.6. After adding 30 µl of 1M dithiothreitol (DDT), the sample was reduced at 54°C overnight. After

adding 60 µl of a 2M solution of sodium iodoacetate, the sample was incubated in the dark for 30 min. After that, there followed a dialysis against 0.5M urea/0.1M ammonium hydrogen carbonate (complete buffer exchange after 3 h and overnight; dialysis bag having a 25 kD exclusion). The sample which had been pretreated in this way was digested at 37°C for 18 h in the presence of 20 µg of trypsin (sequence grade). The sample was concentrated down to approx. 100 µl by drying in a centrifuge.

HPLC separation of the tryptic peptides: One third of the sample was loaded onto an RP-18 column (2.1 mm × 100 mm; 5 µ material) and separated using a Smart System (solution A: 0.1% TFA, solution B: 0.1% TFA/60% ACN; detection: 215 nm, flow: 0.15 ml/min, room temperature; gradient: 7 min 0% B, 52 min, 70%, 54 min 100% B). Because of the high molecular weight of the AT, a very complex mixture is formed during the trypsin digestion. Rechromatography of individual fractions is therefore a prerequisite for obtaining clean peptides for the subsequent sequencing.

Rechromatography of separation fractions: The fractions containing the peptides were pooled and concentrated by drying in a centrifuge. The concentrates were rechromatographed on an RP-18 column (2.1 mm × 100 mm; 5µ material) (solution A: 0.025M NH_4Ac, solution B: 0.025M NH_4Ac/60% ACN, pH 6; detection: 215 nm, flow: 0.15 ml/min, room temperature; gradient: 0 min 0% B, 33 min 60%, 38 min 100% B). After rechromatographing fractions 28 + 29 (Smar 4003) and fraction 30 (Smar 4002) from the first separation using different chromatography conditions, the purity of the resulting peptides was adequate for sequencing the N-terminal sequences.

Sequencing the N-terminal sequences: For example, fraction 32 of the rechromatography (Smar 4002) was evaporated down by drying in a centrifuge. The peptide was dissolved in TFA and, for the sequencing, loaded onto glass fibre filters which had been previously treated with BioBrene®. The peptide was sequenced using the "fast-normal" sequencer cycle. The PTH amino acids were identified and determined quantitatively using the 50 pmol PTH standard. The result of the N-terminal sequence analysis is recorded in Tab. 4. A total of 133 amino acids were analyzed from eight tryptic peptides derived from the AT.

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Table 4 N-terminal sequences of the tryptic peptides derived from acarviosyl transferase

1.1. Rechromatography of fraction 28 + 29 (Smar4003)

1.2. Fraction 35 of the rechromatography

1

Asn-Leu-Gly-Val-Gly-Ala-Ile-Trp-Ile-Ser-Pro-His-Val-Asp-Asn-Ile-Asn-Val-Pro-

22 23

Ala-Ala-Gly-(Gly)...

2.1 Rechromatography of fraction 30 (Smar4002)

2.2 Fraction 32 of the rechromatography

1

Thr-Gly-Lys-Pro-Val-Pro-Val-Gln-Phe-Thr-Val-Gln-Asn-Pro-Pro-Ala-Thr-Ala-Pro-

21

Gly-Glu...

2.3 Rechromatography of fraction 25 (Smar4004)

2.3.1 Fraction 31 of the rechromatography

1 18

Ser-Thr-Val-Ala-Pro-Val-Leu-Gly-Ala-Gly-Gln-Val-Ala-Val-Trp-Ser-Tyr-Arg

2.3.2. Fraction 25 + 26 of the rechromatography

1

Tyr-Gln-Asp-Gln-Tyr-Tyr-Ser-Leu-Ala-Asp-Ile-Ala-Asp-Leu-Asp-Gln-Gln-Asn-

20

Pro-(Arg)

2.4 Rechromatography of fraction 21 (Smar4005)

2.4.1. Fraction 23 of the rechromatography

1 12

Trp-Ile-Asn-Asp-Asp-Val-Tyr-Val-Tyr-Glu-Arg-Leu...

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2.5 Rechromatography of fractions 31 + 32 (Smar4001)

2.5.1 Fraction 30 of the rechromatography

1 18

Asp-Tyr-Leu-Tyr-Glu-Gln-Asp-Leu-Ile-Thr-Phe-Leu-Asp-Asn-Gln-Asp-Thr-Arg

2.6 Rechromatograpy of fractions 16 + 17 (Smar4007)

2.6.1 Fraction 17 of the rechromatography

1 9

Asp-Asp-Ala-Asn-Tyr-Trp-Met-Asp-Arg

2.7 Rechromatography of fraction 20 (Smar4007)

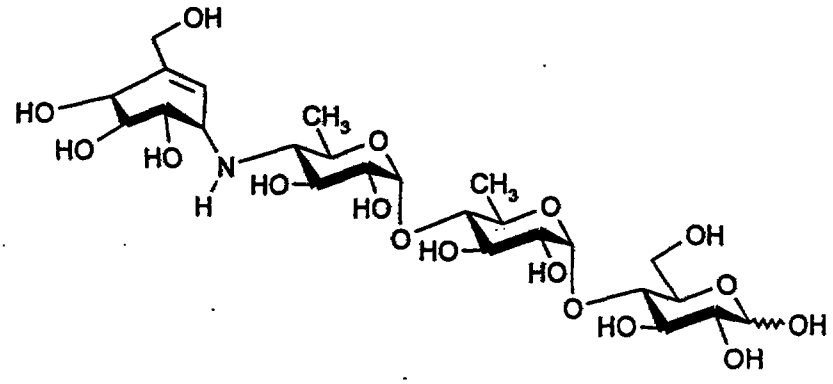
2.7.1 Fraction 11 of the rechromatography

1 12

Ala-Val-Leu-Thr-Gly-Asn-Thr-Val-Tyr-Asp-Trp-Lys

3. Conversion of acarbose homologues into acarbose.

In the investigations on the donor specificity of AT, acarbose homologues, such as the acarbose minor constituents 2, 4A, 4B and 4C and components B and pseudoacarbose



were treated, in experimental mixtures, with maltose in the presence of acarviosyl transferase. After a reaction period of 24 h at 30°C, the experimental mixtures were analyzed by HPLC on amino phase using UV detection. The evaluation (Tab. 5)

demonstrates that the content of minor constituents decreases whereas the content of acarbose increases, i.e. there is a transfer of the acarviosyl unit from the acarbose minor constituents 2, 4A, 4B and 4C to maltose.

Table 5 The use of AT to convert the acarbose minor constituents 2, 4A, 4B and 4C into acarbose

Donors	Reaction time (h)	HPLC analysis (g/l)				
		Acarbose	Comp. 2	Comp. 4A	Comp. 4B	Comp. 4C
Comp. 2	0 24	0.29 0.40	0.60 0.40			
Mixture Comp. 4A, Comp. 4B, Comp. 4C	0 24	0.53 1.70		0.92 0.08	0.78 0.07	0.48 0.26

Traces of a conversion are found with component B and pseudoacarbose.

4. Isolation of higher acarbose homologues from acarbose

In the investigations on the acceptor specificity of AT, acarbose was reacted in experimental mixtures with acarviosyl transferase in the presence of adequately high concentrations of maltooligosaccharides and other sugars. After a reaction period of 18 h at 30°C, the experimental mixtures were analyzed by HPLC on amino phase using UV detection. The evaluation (Tab. 6) demonstrates that newly synthesized saccharides are detected while maltose is concomitantly released.

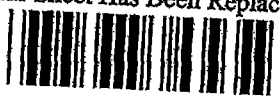
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Table 6 Transfer of the acarviosyl residue of acarbose to different sugars acting as acceptors (PA = percentage area; RT = retention time)

Acceptors	Acarbose (RT)	Acceptor (RT)	Maltose (PA %)	New constituents (PA %)	New constituents (RT)
Demin. water	31.4		2.9	0	
Cellobiose	31.3	13.5	17.2	29	25.0
Deoxy-D-glucose	30.4	4.5	2.6	1	6.6
Gluconic acid lactone	30.6	17.1	3.7	4	37.4
D-Glucose	30.2	5.7	7.6	11	18.0
Isomaltose	31.8	10.1	9.7	16	19.6
Isomaltotriose	30.9	13.9	5.4	7	23.5
Laminaribiose	28.5	15.6	16.3	27	30.1
Maltose	30.2	15.8	38.1		
Maltotriose	31.8	26.8	19.8	21	38.8
Maltotetraose	31.1	38.1	19.9	20	40.6
Maltopentaose	32.6	40.6	17.9	20	42.1
Maltohexaose	31.2	41.7	17.2	15	43
Maltoheptaose	30.3	42.6	18.9	20	43.6
Methyl-D-glucopyranoside	29.2	1.8	11.5	13	3.8
Palatinose	31.1	13.5	9.3	9	24.8
Panose	31.1	21.2	14	20	37.5
Sophorose	30.5	16.5	16.3	34	33.2
Xylobiose	28.5	10.8	20.8	22 / 5	16.7/17.3
D-Xylose	30.7	5.9	5.7	6	14.8
L-Xylose	29.1	5.8	11	15	16.0

When the AT activity was determined in the radioactivity test, an exchange was also observed with dextrin:

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Oligosaccharide	relative Activity (%)
maltose	100
maltotriose	27
maltotetraose	40
maltoheptaose	49
cellobiose	15
dextrin	45

5. Modified process for working up acarbose using AT:

In principle, the reaction catalyzed by AT can also be used to enrich acarbose from culture solutions while at the same time converting acarbose homologues into acarbose, in accordance with the following principle:

1) Reacting acarbose or acarbose homologues [acarviosyl-$(G)_n$] with high molecular weight dextrins or starch [$(G)_m$] in the presence of acarviosyl transferase

acarviosyl-$(G)_n$ + $(G)_m$ → acarviosyl-$(G)_m$ + $(G)_n$

and removing low molecular weight impurities by dialyzing or precipitating the polysaccharides; and then

2) Reacting with maltose, with the liberation of acarbose,

acarviosyl-$(G)_m$ + maltose → acarbose + $(G)_m$

The same effect can also be achieved by way of a reactor (e.g. column) using starch and immobilized AT:

- filtering crude acarbose solution through a starch/AT column
- washing to remove impurities
- eluting acarbose with maltose

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In the above reaction scheme, G represents glucose and m and n in each case represent an integer between 1 and 20, with m and n being different.

6. Culture of E. coli strains, preparation of the plasmid DNA and isolation of DNA fragments

E. coli DH5α was incubated at 37°C in LB medium. Plasmid-harbouring bacteria were maintained under selection pressure (Ampicillin, 100 µg/ml). The cultivation took place on an orbital shaker at 270 rpm. Mixtures which were incubated for at least 16 h were designated overnight cultures (OC).

The cells from 1.5 ml of an OC which had been incubated under selection pressure were employed for preparing plasmid DNA. The plasmids were isolated using the alkaline SDS lysis method (Birnboim and Doly, 1979).

Restriction endonucleases were employed for the specific hydrolysis of vector DNA exclusively in accordance with the manufacturer's instructions (Gibco BRL, Eggenstein, Germany). 5 U of the relevant restriction endonuclease were employed for restricting 10 µg of plasmid DNA, with the incubation being carried out at 37°C for 2 h. In order to ensure complete hydrolysis, the same quantity of restriction endonuclease was added a second time and the mixture was incubated once again for at least 1 h.

Depending on the sizes of DNA fragments, the cleaved DNA was separated electrophoretically on 0.5-1.2% horizontal agarose gels. For eluting the DNA, the gel piece which contained the DNA fragment was excized with a sterile scalpel and weighed. The DNA fragment was then eluted from the agarose using the JETsorb kit in accordance with the manufacturer's instructions (Genomed, Bad Oeynhausen, Germany).

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7. Growth of Actinoplanes sp. SE 50/110, preparation, cleavage of the chromosomal DNA and gel-electrophoretic separation

Actinoplanes sp. SE 50/110 was incubated at 30°C in TSB medium for 3 d on an orbital shaker. While the seed culture (5 ml) was carried out at 240 rpm in small culture tubes, the main culture (50 ml) was carried out at 100 rpm in 500 ml baffled flasks. After the cultivation, the cells were sedimented by centrifugation and washed twice in TE buffer.

The total DNA was prepared by the phenol/chloroform extraction method (Hopwood et al., 1985) using 1.5-2 mg of cells (fresh weight). The 20 µg of chromosomal DNA were hydrolyzed at 37°C for 2 h with 10 U of the relevant restriction enzyme (Gibco BRL, Eggenstein, Germany) in the appropriate buffer. In order to ensure complete hydrolysis, the same quantity of restriction endonuclease was added a second time and the mixture was incubated once again for at least 1 h.

The cleaved DNA was separated electrophoretically on 0.6% horizontal agarose gels.

The DNA fragments were eluted once again using the JETsorb kit (see Example 6).

8. Preparation of the acb gene probe II

The fragment from pAS2 which was prepared as described in Example 6 was radioactively labelled with the nick-translation system supplied by Gibco BRL, Eggenstein, Germany, in accordance with the latter's instructions. 0.5 - 1.0 µg of DNA fragment was employed for this purpose. [α ^{32}P]dCTP was employed (3000 Ci/mM; Amersham, Braunschweig, Germany). The mixture was then boiled for 10 minutes (denaturation) and immediately added to the hybridization solution (see Example 9).

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9. Transfer of DNA to membranes, hybridization of DNA (Southern hybridization) and autoradiography

DNA fragments were transferred from agarose gels to membranes by the Southern transfer method (Southern, 1975). The agarose gels, which were obtained as described in Example 7, were rinsed for 20 minutes in 0.25 M HCl. The gels were laid on three layers of 3MM Whatman paper (Whatman, Maidstone, England), and a Hybond™-N+ membrane (Amersham, Braunschweig, Germany) was laid on the gel in such a way that no air bubbles were formed. Several layers of absorbent paper were then laid on the membrane. A weight of approx. 1 kg was then placed on the filter stack. The DNA was transferred by sucking through 0.4M NaOH. After a transfer time of at least 12 h, the nylon filters were rinsed with 2 × SSC for 5 minutes and dried in air.

The nylon filters were then shaken at 68°C for at least 2 h in 50-100 ml of prehybridization solution in a water bath. During this period, the solution was changed at least twice. The hybridization was carried out in a hybridization oven for at least 12 h. 15 ml of hybridization solution, containing the acb probe II (see Example 8), were employed.

The nylon filters were subsequently washed for in each case 15 minutes with 6 × Postwash and 1 × Postwash. The nylon filters were then covered, while still moist, with airtight film. The autoradiography was carried out, at -80°C for at least 16 h, with Hyperfilm-MP (Amersham, Braunschweig, Germany) in a lightproof cassette using intensifying screens.

10. Isolation and cloning of SstI fragments from the total DNA of Actinoplanes sp. SE 50/110

Actinoplanes sp. chromosomal DNA was completely hydrolyzed with SstI and separated by agarose gel electrophoresis, and the DNA fragments of 9.0 to 12 kb in length were eluted from the agarose (see Example 6). The vector plasmid pUC18 was prepared from E. coli DH5α, hydrolyzed with SstI and treated with alkaline

phosphatase (Boehringer, Mannheim, Germany) in accordance with the manufacturer's instructions. The ligation was carried out in a volume of 20 µl, with the ratio of fragment to vector being 3:1, with 0.01-0.1 µg of DNA being present in the mixture. 1 U of the T4 DNA ligase was employed together with the appropriate buffer (Gibco BRL, Eggenstein, Germany).

E. coli DH5α cells which were transformation-competent were transformed with complete ligation mixtures (in accordance with Hanahan, 1983). Ampicillin-resistant transformants were transferred to LB-Amp selection plates (ampicillin, 100 µg/ml).

11. Identification of plasmids which contain the 11 kb SstI fragment from the acarbose biosynthesis cluster

Ampicillin-resistant transformants were examined for the presence of the 11 kb SstI fragment which hybridizes with the acb probe II. In each case ten of these clones were streaked out on a selection plate, incubated overnight and washed from the plate with 3 ml of LB medium. The plasmid DNA was then isolated from 20 such pools of ten (using the method of Birnboim and Doly, 1979). In order to remove the cloned SstI fragments from the polylinker, the 20 different plasmid preparations were hydrolyzed with the restriction endonucleases EcoRI and HindIII. The restriction mixtures were then fractionated electrophoretically on a 0.6% agarose gel, and the DNA was transferred from the agarose gel to a nylon filter by means of Southern transfer (see Example 9). Hybridization took place once again using the acb probe II (see Example 9). One of the pools reacted positively with the acb probe II and was divided into the ten individual clones. The plasmids of these clones were likewise isolated and subjected to the above-described method. The plasmid which was hybridized was designated pAS5. It contains a 10.65 kb SstI fragment.

12. Cloning of the 2.6 kb HindIII/PstI fragment

In order to be able to identify additional reading frames, several HindIII/PstI subclones were prepared from plasmid pAS5. For this purpose, plasmid pAS5 was hydrolyzed

with the restriction endonucleases HindIII and PstI. The following fragments were obtained:

1.4 kb PstI fragment

5.4 kb PstI fragment

0.05 kb PstI/HindIII fragment

2.6 kb HindIII/PstI fragment

3.8 kb PstI fragment (1.1 kb PstI/SstI fragment ligated to the pUC18 vector)

The resulting DNA fragments were separated by agarose gel electrophoresis and eluted from the gel (see Example 6). The pUC18 vector plasmid was prepared from E. coli DH5α, hydrolyzed with HindIII and PstI and treated with alkaline phosphatase (Boehringer, Mannheim, Germany) in accordance with the manufacturer's instructions. The 2.6 kb HindIII/PstI fragment was cloned. The ligation and transformation were carried out as described in Example 10. The plasmid possessing the 2.6 kb HindIII/PstI fragment was given the designation pAS5/15.1.

13. Amplification and cloning of two 0.3 kb DNA fragments from Actinoplanes sp. chromosomal DNA

In order to sequence the overlapping DNA region between the DNA segments cloned in plasmids pAS5/15.5 and pAS5/15.6, two primers (acbD3 and acbD4) were synthesized from the known DNA sequences of these plasmids (see Example 14). Using these primers, a 0.3 kb DNA fragment was amplified from Actinoplanes sp. chromosomal DNA. The denaturing temperature was 95°C (1 min), the annealing temperature was 68°C (20 sec), and primer extension took place at 72°C (20 sec). 25 amplification cycles were carried out. The Taq polymerase was employed in accordance with the manufacturer's directions (Gibco BRL, Eggenstein, Germany). The PCR mixture contained 5% formamide. The BIOMETRA Personal Cycler (Göttingen, Germany) was used for the PCR reaction. The PCR mixture was precipitated with ethanol and the DNA was subsequently ligated into pUC18 (hydrolyzed with HindIII), and this recombinant plasmid was cloned into E. coli DH5α. In order to sequence the overlapping DNA region between plasmids pAS5/15.4 and pAS5/15.2, a further 0.3 kb

DNA fragment was amplified by means of primers acbD5 and acbD6 using the same experimental mixture, and then cloned.

pAS5/15.18 and pAS5/15.19. After the cloning, the PCR fragment which was amplified with primers acbD3 and acbD4 gave rise to the subclone pAS5/15.18, while the PCR fragment which was amplified with primers acbD5 and acbD6 gave rise to the subclone pAS5/15.19.

14. Subcloning fragments from plasmid pAS5

Several fragments were subcloned from plasmid pAS5 in order to elucidate the sequence of the double stranded DNA (Fig. 1).

pAS5/15.1. Plasmid pAS5 was hydrolyzed with the restriction enzymes HindIII and PstI. Five fragments were produced (see Example 12) of which the 2.6 kb HindIII/PstI fragment was cloned. For this purpose, the restriction mixture was separated on a 0.7% agarose gel, and the 2.6 kb HindIII/PstI fragment was eluted from the gel (see Example 6) and ligated into pUC18 (hydrolyzed with HindIII/PstI), and this recombinant plasmid was cloned into E. coli DH5α.

pAS5/15.2; pAS5/15.3; pAS5/15.4; pAS5/15.5. Plasmid pAS5/15.1 was hydrolyzed with the restriction enzyme SalI. The resulting 6 fragments were separated on a 1% agarose gel. The fragments had the following sizes: 0.75 kb, 0.5 kb, 0.4 kb, 0.35 kb, 0.05 kb and 3.2 kb (0.5 kb fragment ligated to pUC18). The fragments which were earmarked for the subcloning were eluted from the gel (see Example 6). The pUC18 vector was prepared for the cloning using the restriction enzyme SalI as described in Example 6. The ligations were carried out as described in Example 10. The 0.75 kb fragment was ligated into the prepared pUC18, resulting in plasmid pAS5/15.2. Plasmid pAS5/15.3 was obtained after ligating the 0.5 kb fragment to the prepared pUC18. Plasmid pAS5/15.4 contains the 0.4 kb fragment, and the 0.35 kb fragment is a component of plasmid pAS5/15.5.

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pAS5/15.6; pAS5/15.7; pAS5/15.9. Plasmid pAS5/15.1 was hydrolyzed with the restriction enzyme PvuII. The 5 fragments which resulted were separated on a 1.2% agarose gel. The fragments had the following sizes:

1.25 kb PvuII fragment
0.15 kb PvuII fragment
0.8 kb PvuII fragment (0.7 kb PvyII/HindIII fragment ligated to 0.1 kb HindIII/PvuII fragment from pUC18)
0.66 kb PvuII fragment (0.5 kb PvuII/PstI fragment ligated to 0.16 kb PstI/PvuII fragment from pUC18)
2.4 kb PvuII fragment (the residue of the pUC18 vector)

The 1.25 kb fragment was ligated into pUC18 (hydrolyzed with HincII), and this recombinant plasmid was cloned in E. coli DH5α, giving rise to plasmid pAS5/15.6. Plasmid pAS5/15.7 was obtained after cloning the 0.8 kb fragment into the HincII-hydrolyzed pUC18 vector plasmid. Plasmid pAS5/15.9 contains the 0.15 kb fragment.

pAS5/15.12. Plasmid pAS5/15.1 was hydrolyzed with the restriction endonucleases NcoI and KpnI. The resulting 0.9 kb NcoI/KpnI fragment was eluted from a 1.2% agarose gel (see Example 6) and ligated into the vector pUCBM21 (hydrolyzed with NcoI/KpnI), and this recombinant vector was cloned in E. coli DH5α; this resulted in plasmid pAS5/15.12.

15. DNA sequencing of the Actinoplanes sp. 2.6 kb HindIII/PstI fragment

The plasmids described in Examples 13 and 14 were sequenced. 6-8 µg of plasmid DNA from one preparation (see Example 6) were employed in the sequencing reaction. The sequencing reaction was carried out using the Auto-Read Sequencing kit (Pharmacia, Freiburg, Germany). The standard protocol for sequencing dsDNA was used. In order to make it possible to evaluate the nucleotide sequence using the A.L.F., the fluorescein-labelled universal and reverse sequencing primers were used as the starting molecules for the sequencing reaction (see Tab. 3). For preparing the gel, 8 ml

of Hydro Link Long Ranger (Serva, Heidelberg, Germany), 33.6 g of urea, 8 ml of 10× TBE buffer and H_2O to 80 ml were mixed, and the mixture was sterilized by filtration and degassed for 1 minute. Polymerization was initiated by adding 350 µl of 10% (w/v) ammonium persulphate and 40 µl of N,N,N',N'-tetramethylethylenediamine. The solution was poured into a gel form (50 × 50 × 0.05 cm). The electrophoresis was carried out at 38 W at a constant temperature of 45°C. 1 × TBE buffer was used as the running buffer. The measured fluorescence was processed into a DNA sequence using a linked-in computer (Compaq 386/20e), which was also used to control the electrophoresis unit (A.L.F. Manager 2.5 program; Pharmacia).

16. Overexpression of acarviosyl transferase in S. lividans

The gene for Actinoplanes sp. acarviosyl transferase (acbD) was expressed in Streptomyces lividans TK21 from the shuttle vector pUWL201 (U. Wehmeier, unpublished, Fig. 2). The plasmid (6.4 kb) is composed of the vector pUWL199 (Wehmeier, 1995) in which the 2.0 kb KpnI/XbaI fragment is replaced by a KpnI/XbaI fragment consisting of the ermE*p promoter (Bipp et al., 1994) and the HincII/ClaI moiety of the pBLUESCRIPT multilinker (Stratagene). For cloning the acbD gene in E. coli DH5α and Streptomyces lividans TK21, plasmid pAS5/15.1 (see Example 12) was hydrolyzed with the restriction endonucleases HindIII and PstI. The resulting 2.6 kb HindIII/PstI fragment was ligated into vector pUWL201 (hydrolyzed with HindIII and PstI), and the resulting recombinant plasmid was cloned in E. coli DH5α. The resulting plasmid was given the designation pAS9. Plasmid pAS9 was prepared from E. coli DH5α by alkaline lysis and cloned into S. lividans TK21 using the protoplast transformation method (Hopwood et al., 1985). In this clone, the acbD gene is under the control of the constitutive ermE*p promoter (M. Bibb, Norwich, England; personal communication). After S. lividans TK21/pAS9 had been cultured in TSB medium (25 µg/ml thiostreptone), it was possible to detect an additional protein in the supernatant as a significant band of 75 kDa on an SDS polyacrylamide gel (Lugtenberg et al., 1975).

17. Inactivation of the acarviosyl transferase by means of gene disruption

The Actinoplanes sp. acarviosyl transferase gene (acbD) was inactivated by the method of gene disruption. For this purpose, the chromosomal acbD gene in Actinoplanes sp. was partially replaced by an antibiotic resistance gene. The antibiotic resistance genes were inserted by homologous recombination. It was demonstrated in preliminary experiments that Actinoplanes sp. was sensitive to the antiobiotics erythromycin, streptomycin, apramycin, neomycin and kanamycin. The antibiotic resistance genes ermE, aphD1, aaC4 and aph were therefore employed for the mutagenesis. In a first example, the gene for resistance to erythromycin (ermE) from the vector pUGT1 (Ingham et al., 1995) was used for inactivating acbD. For this, the resistance gene, on a 1.5 kb-sized Kpn1 fragIent, was separated on an agarose gel, after having hydrolyzed pUGT1 with KpnI, and then isolated from the gel. Plasmid pAS5/15.1 (see Example 12) was linearized with the restriction endonuclease NcoI. The NcoI recognition sequence is at 1050 bp on the 2.6 kb cloned chromosomal fragment. The hydrolyzed ends of plasmid pAS5/15.1 and of the prepared 1.5 kb KpnI fragment were converted into smooth DNA double-stranded ends with the Klenow fragment of DNA polymerase I in accordance with the producer's instructions (Gibco BRL, Eggenstein, Germany). By means of the subsequent ligation, the erythromycin resistance gene, which is present on the 1.5 kb KpnI fragment, was cloned into the acbD gene on plasmid pAS5/15.1 in E. coli DH5α. This plasmid was linearized and introduced using customary methods (protoplast transformation). The recombinant plasmid can also be transferred by conjugation with E. coli S17-1 and Actinoplanes sp. The method of electroporation constitutes an additional option for plasmid transfer. The chromosomal acbD gene was replaced by the erythromycin resistance gene-interrupted acbD gene of the constructed plasmid as a result of homologous recombination. Due to a double crossover having taken place, an erythromycin-resistant, acbD mutant of Actinoplanes sp. SE 50/110 was produced. The following methods can also be employed for recombining alternative resistance genes into Actinoplanes sp.: (1) electroporation, (2) protoplast transformation (Hopwood et al., 1985), (3) mycelial transformation (Madon and Hutter, 1991) and (4) conjugation (Mazodier et al., 1989).

Buffers and solutions:

Media for growing bacteria

LB medium:

tryptone	10 g
NaCl	10 g
yeast extract	5 g
H_2O	to 1000 ml

The pH was adjusted to 7.5 with 4M NaOH

TSB medium:

tryptone-soya broth (oxoid)	30 g
H_2O	to 1000 ml

TE buffer (pH 8.0)

Tris-HCl	10 mM
Na_2-EDTA	1 mM

Standard preparation of plasmid DNA
(modified from Birnboim and Doly, 1979)

Mix I	50 mM glucose
	50 mM Tris-HCl (pH 8.0)
	10 mM EDTA (pH 8.0)
	5 mg of lysozyme/ml
Mix II	200 ml of NaOH
	1% (w/v) SDS (sodium dodecyl sulphate)
Mix III	3 M potassium acetate
	1.8 M formate

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DNA/DNA hybridization

20× SSC 3 M NaCl

0.3 M Na citrate

prehybridization solution:

6× SSC

0.01 M sodium phosphate buffer, pH 6.8

1 mM EDTA

0.5% SDS

0.1% skimmed milk powder

hybridization solution:

The acb probe is added, after the labelling reaction, to 15 ml of prehybridization solution.

6× Postwash

6× SSC

0.5% SDS

DNA sequencing

TBE buffer (pH 8.0)

1 M Tris base

0.83 M boric acid

10 mM EDTA

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Patent Claims

1. Acarviosyl transferase.

2. Acarviosyl transferase from Actinoplanes sp. SE 50 or SE 50/13 or SE 50/110, and mutants.

3. Essentially pure acarviosyl transferase.

4. Acarviosyl transferase having the amino acid sequence in Fig. 3, and its functional derivatives.

5. DNA sequence in Fig. 4 encoding acarviosyl transferase and its functional derivatives.

6. Process for converting acarbose minor constituents into acarbose,
acarviosyl-G_n + maltose → acarbose + G_n, in which
G_n represents glucose, disaccharides and maltooligosaccharides, characterized in that the reaction is catalyzed by an acarviosyl transferase.

7. Use of acarviosyl transferase in the conversion of other pseudooligosaccharides, such as trestatin or amylostatin, into acarbose, or of other glucosidase inhibitors.

8. Use of acarviosyl transferase for enriching acarbose from cultured supernatants while at the same time converting acarbose homologues into acarbose by reacting with high molecular weight dextrins or starch in the presence of acarviosyl transferase,
acarviosyl-$(G)_n$ + $(G)_m$ → acarviosyl-$(G)_m$ + $(G)_n$,
in which
G represents glucose and m and n denote an integer between 1 and 20, with m and n in each case being different,

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and removing low molecular weight impurities by means of dialysis or reverse osmosis or by precipitating the polysaccharide, and subsequently reacting with maltose, with the liberation of acarbose,

acarviosyl-$(G)_m$ + maltose $\rightarrow$ acarbose + $(G)_m$,

in the presence of adequately high concentrations of maltose, using acarviosyl transferase.

9. Recombinant preparation of acarviosyl transferase in a heterologous host organism.

10. Vector containing the DNA according to Claim 5.

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