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**Recombinant L-N-carbamoylase from
Arthrobacter aurescens, process for the preparation of L-
amino acids by means of this**

The present invention relates to a new recombinant (rec-) L-N-carbamoylase from *Arthrobacter aurescens* and to a process for the preparation of L-amino acids by means of this carbamoylase.

The L-amino acids which can advantageously be prepared by means of the new rec-carbamoylase are important chiral starting materials for the organic synthesis e. g. of drugs, or are of importance in human and animal nutrition.

The advantageous possibility of stereoselective cleavage by L-N- or D-N-carbamoylases of the D,L-carbamoylamino acids which are accessible from the corresponding hydantoins by means of hydantoinases has hitherto led to the establishing of processes for the preparation of L- and D-amino acids on an industrial scale. Evidence of the presence of L-specific carbamoylases has been provided for the most diverse strains of microorganism (Ogawa et al., J. Mol. Catal. B: Enzym. 1997, 2, 163-176; Syltatk et al. in Drauz, K. and Waldmann, H.: Enzyme Catalysis in Organic Synthesis, Weinheim: VCH-Verlag, 1995).

The presence of L-N-carbamoylase activity in cells of the strain *Arthrobacter aurescens* has also already been known for a relatively long time. There has moreover been success in isolating this carbamoylase in a homogeneous form and in determining the N-terminal sequence (for lit. see the article; Müller, doctorate thesis TU Braunschweig 1990). However, the gene which codes for the enzyme was unknown and accordingly has so far been neither cloned nor over-expressed. The recombinant enzyme and the amino acid sequence thereof or the DNA which codes this amino acid sequence were thus not known in the literature.



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Three L-carbamoylases prepared by recombinant methods are so far to be found in the literature (Batisse et al., Appl. Environ. Microbiol. 1997, 63, 763-766; Mukohara et al., Biosci. Biotechn. Biochem. 1993, 57, 1935-1937; Watabe et al., J. Bacteriology 1992, 174, 962-969). However, the L-carbamoylases mentioned there are relatively unstable and are thus unsuitable for use in a large-scale industrial process.

It has hitherto been possible to employ the L-specific carbamoylase from *Arthrobacter aurescens* (A.a.) for cleavage of N-carbamoylamino acids, to give the free L-amino acids, only as a predominantly homogeneously purified enzyme or in the form of free or immobilized dormant whole cells. Some fundamental problems arise in the latter process. These include, inter alia, transport limitations (especially for the N-carbamoyl-amino acids), possible side reactions (the amino acids formed being degraded by further enzymes present) and impurities which contaminate the product by lysis of the whole cells used and necessitate expensive purification procedures.

In both the forms employed, the L-carbamoylase from A. a. is very unstable, which in the case of the use of dormant immobilized forms is attributed to the natural protein turnover of the enzyme (Siemann, doctorate thesis TU Braunschweig 1992). In the case of the use as a homogeneously purified enzyme, the lability is evidently due to unavoidable changes in the protein concentration and the salt content. The sensitivity of the wild-type enzyme to oxidation reactions has furthermore been observed. Moreover, when the carbamoylase from the wild-type strain is purified, only a very low yield of 2.7% is achieved (Müller doctorate thesis TU Braunschweig 1990). This low yield and the necessary complicated purification process as well as the hitherto inherent lability of the enzyme are prohibitive for use in a competitive industrial process for the production of L-amino acids.

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The object of the present invention was therefore to obtain more stable L-carbamoylase in a simpler form and with a better yield compared with the L-carbamoylases of the prior art and with those which can be prepared to date from

5 Arthrobacter aurescens. Nevertheless, the further requirements which a process on an industrial scale imposes on the enzyme employed, such as e. g. activity or selectivity with respect to the substrate and stereochemistry etc., should not be adversely influenced.

10 These and further objects which are not mentioned in more detail but which can readily be seen from the prior art are achieved by enzymes which are the subject matter of claim 1. The amino acid sequences thereof are protected in claim 2, and the gene sequences derived therefrom are protected
15 in claim 3. Claim 4 relates to the vectors modified with the gene sequences, claim 5 to the corresponding plasmids and claim 6 to the modified guest organisms and cells of the guest organisms.

Claims 7 to 10 comprise an advantageous process for the
20 preparation of L-amino acids using these enzymes according to the invention.

By preparing L-N-carbamoylase from Arthrobacter aurescens or mutants thereof by a recombinant method, L-carbamoylases which, compared with the carbamoylases of the prior art,
25 have a far higher stability with nevertheless a good activity and very good stereoselectivity with respect to the biotransformation in question are arrived at in very good yields of >90 % and a decidedly high purity. These facts are essential for the successful use of the
30 carbamoylases in a large-scale industrial process for the preparation of L-amino acids.

It has been found in stability studies that homogeneously purified carbamoylase has an activity for only a few minutes at 50 °C (Müller doctorate thesis TU Braunschweig
35 1990), while the rec-carbamoylase is active for hours. At

37 ° C, the activity of the rec-carbamoylase is almost unchanged for 100 h (fig. 2). This was in no way to be foreseen, and is nevertheless all the more advantageous.

The use of these recombinant carbamoylases moreover opens up for the first time the possibility of obtaining a large-scale industrial access to β -aryl-substituted L-amino acids by an enzymatic biotransformation via (D,L)-N-carbamoylamino acids. The carbamoylase in question, as the only one of the carbamoylases known to date, also offers the possibility of conversion of β -aryl-substituted L-N-carbamoylamino acids to a degree adequate for a large-scale industrial process, in addition to the high L-enantioselectivity. (D,L)-Formylamino acids of this origin are also suitable for reaction with the enzymes according to the invention. The free L-amino acids are likewise preferentially obtained from these.

The amino acid sequences which characterize the recombinant L-N-carbamoylase from *Arthrobacter aurescens* and mutants thereof coincide to the extent of only a maximum of 38 % with the L-N-carbamoylase amino acid sequences known from the prior art. The new and inventive gene sequences which code these amino acid sequences can be prepared by biochemical methods which are known per se. The vectors, plasmids and guest organisms cells containing these gene sequences are also new and inventive. Preferred plasmids are pAW 16 and pAW 178-2 (fig. 1), a preferred vector is pJOE 2702. Guest cells which can be used are in principle all the microorganisms which are known to the expert and are suitable for this purpose, but *E. coli* JM 109 or *E. coli* W3110 is preferred.

Induction of the expression can in principle be achieved
5 with all the methods known to the expert. However, rhamnose
systems, IPTG systems and lactose systems are preferred.

The L-N-carbamoylase gene *hyuC* from *Arthrobacter aurescens*
is isolated and expressed as follows.

A gene library representative of the entire genome of
10 *Arthrobacter aurescens* in an *E. coli* λ RESIII vector was
compiled and screened with an oligonucleotide which was
derived from the N-terminal amino acid sequence of the
purified hydantoinase from *Arthrobacter aurescens*. The
plasmid pAW16, which contained a 7.6 kb DNA fragment from
15 *Arthrobacter aurescens*, was obtained here. The nucleotide
sequence thereof was determined in its entirety. The
hydantoinase gene was identified from the nucleotide
sequence. A further reading frame, the amino acid sequence
derived from which showed homology to an N-L-carbamoylase
20 from *Pseudomonas* sp. and *Bacillus stearothermophilus*, was
identified at the C-terminal end of the hydantoinase gene
hyuH. The start of the protein derived moreover showed
agreement with the N-terminal end, determined by amino acid
sequencing, of the L-N-carbamoylase purified from
25 *Arthrobacter aurescens*. The carbamoylase gene was amplified
via a PCR from pAW16 and inserted into an *E. coli*
expression vector. Carbamoylase can be produced in a high
amount in the recombinant cells via a promoter which can be
induced by the sugar rhamnose.

30 Sequence protocol Seq ID No 4 of the DNA and Seq ID No 5 of
the peptide reproduce the nucleotide sequence and the amino
acid sequence of the rec-carbamoylase from A.a.

Mutants of L-carbamoylase are understood by the expert as
meaning enzymes which are optionally prepared by exchange

of individual amino acids but are derived from the rec-carbamoylase from A.a. and which, in respect of the characteristics referred to above, are suitable in an equally good or even better manner for use in the process described (better possibility of working up, lower sensitivity to oxidation etc.). Mutants are also understood as meaning those enzymes which are formed by addition of amino acids or amino acid sequences to the rec-enzyme. Those which have a His-TAG or Asp-TAG modification on the C-terminus are preferred here.

According to the invention, the desired L-amino acids are prepared from the corresponding L-N-carbamoyl-amino acids or L-N-formyl-amino acids with these advantageous L-N-carbamoylases. As already stated, the L-N-carbamoylases claimed show preferred conversion of β -aryl-substituted L-N-carbamoyl- or L-N-formylamino acids. These chiral compounds can preferably be prepared from chiral hydantoins via hydantoinases. By the additional use of a chemical or enzymatic process for the racemization of hydantoins, a process which is valuable on a large industrial scale for the production of L-amino acids can be constructed in an extremely advantageous manner from hydantoins which are easy to prepare. Chiral 5-monosubstituted hydantoins with aromatic side chains are preferably suitable here. The stereoisomers of these hydantoins are constantly equilibrated by the prior racemization step - optionally by a racemase - while the hydantoinase brings about the corresponding L- or D,L-N-carbamoylamino acids. The stereospecific rec-L-N-carbamoylases according to the invention then take care of the last step for the preparation of the L-amino acid, since the hydantoinase from A.a., in contrast to the carbamoylase, does not show absolute stereospecificity. The prior constant equilibration of the stereoisomers of the hydantoins ensures that all the hydantoin, whether in the D- or L-configuration, is finally converted into an L-amino acid.

Preferably, the protected amino acids are reacted with the enzymes in a so-called enzyme membrane reactor. The stability of the enzymes employed ensures that the enzymes retained in the reactor by an ultrafiltration membrane can be employed several times (>10 times) in succession for L-amino acid production. Costs and outlay for the process are therefore saved, since it is merely necessary to meter in substrate and draw off product.

Alternatively, the enzyme can be immobilized on carriers. This was successfully achieved in a sufficiently high yield only after it was possible for the rec-enzyme to be employed in a pure form. All the methods known to the expert (covalent, adsorptive etc.) for immobilizing enzymes on carrier materials (silica gels, SiO_2 , EAH-Sepharose, nitrocellulose, Eupergit®) can be used, but carbodiimide-mediated covalent coupling to EAH-Sepharose is advantageous. When modified in this way, the enzyme can also be used in a fixed bed reactor.

The following examples are intended to illustrate the invention, but in no way limit it.

Examples:

Isolation of the whole DNA from *Arthrobacter aurescens*:

180 ml yeast extract medium K2 (Bacto-peptone 4.0 g, yeast extract, 4.0 g, glycine, 7.5 g, KH_2PO_4 2.0 g, $\text{K}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ 4.0 g, pH 7.0 in 1000 ml H_2O) were inoculated with *Arthrobacter aurescens* and incubated at 30 °C with shaking (200 rpm). The cells were harvested by centrifugation in a Sorvall GSA rotor at 4 °C, 6000 rpm for 10 min, and the pellet was washed in 20 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), centrifuged again as before and resuspended

in 16 ml TES buffer (25 mM Tris-HCl, 25 mM EDTA, 10 % sucrose, pH 8.0). For cell lysis, 2 ml lysozyme solution (100 mg/ml TES buffer) were added and the mixture was incubated for 60 min at 37 °C. 2 ml 10 % Na lauryl sarcosinate and 2 ml proteinase K (10 mg/ml) were then added and the mixture was incubated for a further 60 min at 37 °C. 20 g CsCl and 0.6 ml ethidium bromide (10 mg/ml) were then added and the mixture was centrifuged in a Sorvall ultracentrifuge at 35000 rpm, 17 °C , T1270 rotor for 48 hours. The DNA was identified via UV light, drawn off from the centrifuge tube via a disposable syringe and dialysed against 2 litres of TE buffer for 2 h. The ethidium bromide was removed by phenol extraction twice with 2 ml neutralized phenol each time and the DNA solution was dialysed again twice against 2 L TE buffer each time.

Construction of the λ gene library:

10 μ g genomic DNA of *Arthrobacter aurescens* were treated with 0.75 unit of the restriction enzyme XhoII for 30 min at 37 °C. The genomic DNA was only partly cleaved here. The DNA was separated in a 0.7% "low melting agarose gel", the DNA was visualized by UV light and an agarose block with fragments of between 7 kb to 13 kb in size was cut out. The agarose block was melted at 65 °C in a volume of 500 μ l buffer (TE buffer with 0.5 M NaCl), extracted with the same amount of phenol, and the agarose and phenol were separated off by centrifugation at 4 °C in an Eppendorf centrifuge at 13000 rpm, 30 min, 4 °C. One tenth the volume of 3 M Na acetate, pH 6.2 and 2.5 volumes of ethanol were added to the aqueous phase, the mixture was incubated at -70 °C for 30 min and centrifuged in an Eppendorf centrifuge for 10 min, the liquid was removed and the DNA precipitated was washed with 100 μ l 70 % ethanol, dried in air and resuspended in 10 μ l TE buffer. The λ RESIII vector was prepared as described in Sambrook et al. The phage is a

substitution vector, i. e. a restriction fragment is removed from the vector DNA and replaced by another fragment. Without an insert, the phage is not capable of multiplication (Altenbuchner, 1993). In this case, a 9 kb BamHI fragment with the bacterial Lux genes was removed from the vector by treating 1 µg vector DNA with 10 units of BamHI for 1 h, separating the DNA over "low melting agarose" and purifying the DNA of the λ arms via the gel as described above. The DNAs were then ligated by incubating in each case about 25 ng of the two vector arms with 25 ng of fragments of *A. aurescens* cleaved with XhoII in a volume of 10 µl, ligase buffer of the manufacturer (Boehringer GmbH) and 0.5 unit of ligase overnight at 17 °C. 2 µl of the batch were employed in an in vitro packing system and packed in phage particles. The packing batch was prepared as described in Sambrook et al. from the two *E. coli* strains *E. coli* BHB2690 (sonified extract, SE) and BHB2688 (freeze-thaw extract FE). For packing, 7 µl buffer A (20 mM Tris-HCl, 3 mM MgCl₂, 0.05 % (V/V) 2-mercaptoethanol, 1 mM EDTA, pH 8.0), 10 µl FE extract, 6 µl SE extract, 1 µl buffer M1 (113 µl H₂O, 3 µl 1 M Tris-HCl, pH 7.5, 300 µl 0.05 M spermin, 0.1 M putrescin, 75 µl 0.1 M ATP, 1 µl 2 mercaptoethanol, 9 µl 1 M MgCl₂) and 2 µl DNA from the ligation batch were mixed and incubated for 1 h at 25 °C. The packing was stopped by addition of 0.5 ml SM buffer (5.8 g NaCl, 2 g MgSO₄·7 H₂O, 50 ml 1 M Tris-HCl, pH 7.5, 2 g gelatine, per 1 L) and one drop of toluene.

Identification of a recombinant phage with the hydantoinase gene by plaque hybridization:

The phage lysate obtained from the in vitro packing was diluted to about 5000 phages/ml in SM buffer. 0.1 ml diluted phage lysate was incubated with 0.1 ml of an

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overnight culture in LB_{mal} of the E. coli strain TAP90 for 5 min at room temperature, taken up in 3 ml LB_{mal} soft agar and poured on to an LB plate and incubated at 37 °C overnight. (LB_{mal}: 10 g Bacto-tryptone, 5 g yeast extract, 5 10 g NaCl, 2 g maltose, per 1 litre H₂O, pH 7.5; LB agar plates: as LB_{mal} without maltose with 1.5 % agar, LB_{mal} soft agar as LB_{mal} with 0.7 % agar). Visible plaques developed on the plate in this period of time. A nylon filter (Quiabrone, Quiagen GmbH) was placed on the agar plate and 10 the phage DNA was thereby transferred into the plaques on the filter. The DNA on the filter was denatured for 5 min by placing the filter on a stack of Whatmann paper impregnated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) and renatured for 5 min in the same manner with the 15 renaturing solution (1.5 M NaCl, 0.5 M TrisHCl, pH 7.4). The filter was washed in 2xSSC (1xSSC: 8.75 g NaCl, 4.4 g Na citrate per 1 L H₂O, pH 7.0), dried and baked in a vacuum oven for 30 min at 120 °C in order to fix the DNA. The filter was then prehybridized with hybridizing solution 20 (5xSSC, 1 % blocking reagent from Boehringer Mannheim, 0.01 % Na lauryl sarcosinate, 0.02 % sodium dodecylsulfonate) at 32 °C. After two hours, radioactively labelled oligonucleotide was added. The oligonucleotide was obtained from MWG Biotech GmbH, and the nucleotide sequence 25 was derived from the N-terminal amino acid sequence of the L-hydantoinase from *Arthrobacter aurescens* (Siemann, Dissertation 1992).

Oligonucleotide: 5'-

ATGTT(C/T)GA(T/C)GT(A/C/T/G)AT(A/C/T)GT-3' (Seq. 1)

30 10 pmol of this oligonucleotide were labelled with 80µCi γ-³²P-ATP and 6 units of polynucleotide kinase in a volume of 10 µl for 30 min at 37 °C and the labelled oligonucleotide was added to the hybridization solution. Hybridization was carried out at 32 °C for 20 hours. The filter was washed 35 first briefly with 0.2xSSC, 0.1 % SDS at room temperature, 30 min at 32 °C and a further time with 0.1xSSC, 0.1 % SDS

at room temperature, 30 min at 32 ° C and a further time with 0.1xSSC, 0.1 % SDS for 15 min at 32 ° C. After drying, positive phages were determined via autoradiography. Out of 2500 phages, two which hybridized with the probe were isolated here. One of the phages was investigated further.

Conversion of the recombinant phage into a plasmid:

The vector λ RESIII contains, in addition to the λ -genes and the substitution fragment, a replication origin of the E. coli plasmid RtsI, a kanamycin resistance gene and two recognition sequences (res) for a position-specific recombination system (the resolvase from the transposon Tn1721). The phage is inhibited in its multiplication by infection of a specific E. coli strain (E. coli HB101 F'Lac [::Tn1739 tnpR]) which contains a derivative of this transposon, and at the same time the plasmid is cut out of the phage DNA together with the substitution fragment via the transposon-coded resolvase and converted into a plasmid. Recombinant phages were isolated from the plaque which hybridized with the probe by cutting out the plaque region from the agar plate. The material was resuspended in 0.5 ml SM buffer and 0.1 ml of this phage suspension was mixed with 0.1 ml of an overnight culture of the strain E. coli HB101 F'Lac [::Tn1739 tnpR], the mixture was incubated for 5 min at room temperature and introduced into 2 ml LB medium with 0.1 M isopropyl β -thiogalactopyranoside and the mixture was incubated for 45 min on a roller at 37 ° C. Aliquots were then plated on to LB agar plates with 50 μ g/ml kanamycin and the agar plates were incubated at 37 ° C overnight. Plasmid DNA was isolated from the resulting colonies by a method described by Kieser (1984), and the plasmid pAW16 was thereby obtained. pAW16 contains a fragment 7.6 kb in size from *Arthrobacter*

aurescens. The fragment was sequenced in full with the aid of an ALF sequencers and the AutoRead™ Sequencing Kits from Pharmacia and analysed with the programs of the GCG software package. An open reading frame could be identified as the hyuH gene for hydantoinase on the basis of the identical nature of the amino acid sequence derived with the sequenced N-terminal end of the hydantoinase. Another open reading frame was identified at the end of hyuH, starting with a GTG start codon one base before the stop codon TGA of the hyuH gene. The amino acid sequence derived for this open reading frame shows homology to an N-L-carbamoylase of *Bacillus stearothermophilus* NS1122A (Mukohara et al., 1993) and of *Pseudomonas* sp. NS671 (Watabe et al., 1992). The N-terminal amino acid sequence moreover coincides with the N-terminal sequence of the N-L-carbamoylase from *A.s* [sic] (Siemann Dissertation 1992). The gene was designated hyuC for N-L-carbamoylase.

Expression of hyuC in *E. coli* JM109 via a promoter which can be induced with rhamnose:

For expression of the N-L-carbamoylase gene hyuC in *E. coli*, the gene was amplified via the polymerase chain reaction (PCR). The two primers S956 and S957 were obtained from MWG Biotech GmbH.

S956: 5'-AGAACATATGTTTGACGTAATAGTTAAGAA-3' (Seq. 2)

S957: 5'-AAAAGGATCCTCACTTCGACGCCTCGTA-3' (Seq. 3)

By means of the primer, a restriction cleavage site for the enzyme NdeI was inserted at the N-terminus, and a BamHI cleavage site at the C-terminus. The two cleavage sites are necessary to insert the gene after the rhamnose promoter of the vector pJOE2702 (Volf et al., 1996). The GTG start codon used for translation of the gene in *Arthrobacter aurescens* was furthermore converted into an ATG start codon.

The batch was conducted in 100 μ l. 1 ng pAW16 DNA, in each case 30 pmol of the two primers, 0.2 mM dNTP, 16 mM $(\text{NH}_4)_2\text{SO}_4$, 67 mM Tris-HCl, pH 8.8, 0.01 % Tween, 1.5 mM MgCl_2 , 0.5 unit of Taq polymerase were employed. The amplification was carried out in 30 cycles with three stages, 1 min 94 $^\circ\text{C}$, 1 min 50 $^\circ\text{C}$ and 1.5 min 72 $^\circ\text{C}$ in a MiniCycler PTC150, Biozym Diagnostik GmbH. The amplified fragment was purified over a QUIAquick Spin column (Quiagen GmbH). 500 ng of the fragment were treated in a volume of 20 μ l with in each case 10 units of the restriction enzymes NdeI and BamHI for 1 h in the buffer supplied by the manufacturer. DNA of the vector pJOE2702 was also treated with these enzymes under the same conditions. The DNAs were separated over a 0.7 % agarose gel and the fragments were isolated from the gel as described above. In each case 20 ng PCR fragment and 20 ng vector were ligated under the conditions described above. *E. coli* JM109 was transformed with the ligated DNA in accordance with a protocol of Chung et al., (1989). The cells were plated on to LB agar plates which contained 100 $\mu\text{g/ml}$ ampicillin (LB_{amp}). The following day, plasmids were isolated from ampicillin-resistant colonies and characterized with the restriction enzymes NdeI and BamHI. The plasmid pAW178-2 contained the *hyuC* gene in the desired orientation under the rhamnose promoter.

Expression of *hyuC* in *E. coli* JM109/pAW178-2:

1 ml of an overnight culture of *E. coli* JM109/pAW178-2 (fig. 1) in LB_{amp} is introduced into 100 ml fresh LB_{amp} medium in a 1 litre conical flask and this is incubated at 30 $^\circ\text{C}$ in a water-bath, with shaking (200 rpm). At an optical density of the culture of 0.5 (measured at a wavelength of 600 nm in a photometer), 1 ml of a 20% rhamnose solution is added to the culture (final

concentration 0.2 g/L) and the culture is incubated further. After incubation for a further 9 h, about the maximum of the N-L-carbamoylase activity is reached, a specific activity of 1.8 units/mg crude extract, measured with N-carbamoyl-L-tryptophan as the substrate. No activity was found in cultures which had not been induced with rhamnose.

**Determination of the substrate spectrum of the
rec-L-N-Carbamoylase from A.a.**

The substrate specificity of the carbamoylase was investigated with homogeneously purified enzyme. This was obtained after cell breakdown and ion exchange chromatography on Streamline-DEAE and MonoQ media. A solution of the particular substrate in a concentrations [sic] of 1.8 mM (exception N-carbamoyl-L-methionine: 50 mM) in 0.1 M Tris-HCl, pH 8.5 was prepared and was used instead of the standard substrate in the standard activity test. Instead of the trichloroacetic acid (TCA) used for stopping in the standard test, by means of [sic] heat denaturation (incubation for 10 min in a boiling water-bath) was used in the case of the substrates N-formyl-D,L-tryptophan, N-carbamoyl-L-thienylalanine and N-acetyl-D,L-tryptophan, and in the case of the substrate N-carbamoyl-D,L-phenylalanine 900 µl methanol to 100 µl assay solution were used. The concentration of the amino acid was then determined either by means of HPLC or colorimetrically after reaction with ninhydrin or by HPLC.

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Tab. 1: Determination of the substrate spectrum and the stereospecificity of the rec-L-N-Carbamoylase from A.a.

Substrate	Product	Activity	K _m	Analytical method
		%	[mM]	
N-Carbamoyl-L-tryptophan	L-Tryptophan	100	0.12	HPLC colorimetric
N-Carbamoyl-D-tryptophan	D-Tryptophan	n.d.		HPLC
N-Formyl-D,L-tryptophan	L-Tryptophan	97	8.5	HPLC
N-Acetyl-D,L-tryptophan	L-Tryptophan	n.d.		HPLC
N-Carbamoyl-L-thienylalanine	L-Thienylalanine	314	2.3	HPLC colorimetric
N-Carbamoyl-D,L-phenylalanine	L-Phenylalanine	97	0.7	HPLC
N-Carbamoyl-D-phenylalanine	D-Phenylalanine	n.d.		HPLC
N-Carbamoyl-L-tyrosine	L-Tyrosine	126	0.3	HPLC
N-Carbamoyl-L-methionine	L-Methionine	17		HPLC colorimetric
N-Carbamoyl-L-phenylglycine	L-Phenylglycine	n.d.		HPLC colorimetric
N-Carbamoyl-D-phenylglycine	D-Phenylglycine	n.d.		colorimetric
N-Carbamoyl-β-alanine	β-Alanine	n.d.		colorimetric
N-Carbamoyl D,L-aspartate	L-Aspartate	n.d.		colorimetric
N-Formyl L-aspartate	L-Aspartate	n.d.		colorimetric
N-Carbamoyl L-glutamate	L-Glutamate	n.d.		colorimetric
N-Carbamoyl-L-arginine	L-Arginine	n.d.		colorimetric

n.d.: not detectable

Heat stability of the rec-L-N-Carbamoylase from A.a.

The homogeneously purified recombinant L-carbamoylase was stored at 37 °C. The standard test for determination of activity was in each case carried out at intervals of time of 1 - 100 h. The activity initially measured remained virtually unchanged for 100 h (fig. 2).

Immobilization of the rec-L-N-Carbamoylase:

250 µl enzyme solution (after the purification, protein concentration: 2.3 mg/ml) are diluted with 19.75 ml of a solution of 1 mM MnCl₂ in water (final protein concentration: 0.028 mg/ml). 4 ml of this solution are added to 1.5 g of previously washed EAH-Sepharose 4B (from Pharmacia Biotech, washing successively with 0.1 M phosphate buffer, pH 6.5 and then with a solution of 1 mM MnCl₂ in water). After an adsorption phase of 20 min, 545 µl of a coupling solution of 500 mM N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC) in water, pH 6.0 are added to the batch (final concentration of EDC: 60 mM) and the mixture is shaken in an overhead shaker for up to 30 h (preferably 15 h). The solid is filtered off with suction and washed three times with 0.2 M Tris buffer, 0.5 M NaCl, pH 7.0. The immobilized material is kept in the last washing solution for one hour. It is then washed again (0.1 M Tris buffer, pH 7.0) and kept in 0.1 M Tris buffer, 0.1 mM MnCl₂, pH 7.0). The activity of the immobilized enzyme was 100 %, compared with that of the enzyme employed.

Enzyme measurements of the N-L-Carbamoylase:

5 ml of a cell culture induced with rhamnose are centrifuged at 3000 rpm for 5 min in a Heraeus Megafuge, washed with 5 ml buffer (200 mM Tris-HCl, pH 7.0), centrifuged again, taken up in 1 ml buffer (200 mM Tris-

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HCl, 0.1 mM MnCl_2 pH 7.0) and broken down by ultrasound (Ultrasonics Sonicator, Microtip, 2x30 sec, duty cycle 50 %). Cell debris is removed by centrifugation in an Eppendorf centrifuge at 13000 rpm for 10 min. The

5 supernatant, called the crude extract, is employed for the enzyme measurement. 100 μl crude extract or purified enzyme are mixed with 800 μl 2 mM N-carbamoylamino acid, preheated at 37 °C, in 100 mM Tris-HCl, pH 8.5 and the mixture is incubated for 10 min at 37°C. The reaction is stopped by

10 addition of 400 μl trichloroacetic acid. The protein which has precipitated out is separated off by centrifugation in an Eppendorf centrifuge at 13000 rpm for 10 min. The supernatant is then measured in an HPLC or colorimetrically (ninhydrin). The reaction mixture is separated in an HPLC

15 with an RP-18 column, the mobile phase is 0.3 % (V/V) phosphoric acid, 80 % methanol, flow rate 1.0 ml/min. Detection is by measurement of the UV absorption [sic] at a wavelength of 280 nm.

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SEQUENCE LISTING

<110> Degussa-Hüls AG, Universität Stuttgart, Roche Diagnostics GmbH

<120> Recombinant L-N-carbamoylase from *Arthrobacter*
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by means of this

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gag ctc tcc cgg ttc tcg gca gaa ggc ccc ggt gtt acc cgg ctg acc	96
Glu Leu Ser Arg Phe Ser Ala Glu Gly Pro Gly Val Thr Arg Leu Thr	
20 25 30	
tac act cca gag cat gcc gcc gcg cgg gaa acg ctc att gcg gct atg	144
Tyr Thr Pro Glu His Ala Ala Ala Arg Glu Thr Leu Ile Ala Ala Met	
35 40 45	
aaa gcg gcc gcc ttg agc gtt cgt gaa gac gca ctc gga aac atc atc	192
Lys Ala Ala Ala Leu Ser Val Arg Glu Asp Ala Leu Gly Asn Ile Ile	
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ggc cga cgt gaa ggc act gat ccg gag ctt cct gcg atc gcg gtc ggt	240
Gly Arg Arg Glu Gly Thr Asp Pro Glu Leu Pro Ala Ile Ala Val Gly	
65 70 75 80	
tca cac ttc gat tct gtc cga aac ggc ggg atg ttt gat ggc act gca	288
Ser His Phe Asp Ser Val Arg Asn Gly Gly Met Phe Asp Gly Thr Ala	
85 90 95	
ggc gtg gtg tgc gcc ctt gag gct gcc cgg gtg atg ctg gag aac ggc	336
Gly Val Val Cys Ala Leu Glu Ala Ala Arg Val Met Leu Glu Asn Gly	
100 105 110	
tac gtg aat cgg cat cca ttt gag ttc atc gcg atc gtg gag gag gaa	384
Tyr Val Asn Arg His Pro Phe Glu Phe Ile Ala Ile Val Glu Glu Glu	
115 120 125	
ggg gcc cgc ttc agc agt ggc atg ttg ggc ggc cgg gcc att gca ggg	432
Gly Ala Arg Phe Ser Ser Gly Met Leu Gly Gly Arg Ala Ile Ala Gly	
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ttg gtc gcc gac agg gaa ctg gac tct ttg gtt gat gag gat gga gtg	480
Leu Val Ala Asp Arg Glu Leu Asp Ser Leu Val Asp Glu Asp Gly Val	
145 150 155 160	
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Ser Val Arg Gln Ala Ala Thr Ala Phe Gly Leu Lys Pro Gly Glu Leu	
165 170 175	
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Gln Ala Ala Ala Arg Ser Ala Ala Asp Leu Arg Ala Phe Ile Glu Leu	
180 185 190	
cac att gaa caa gga ccg atc ctc gag cag gag caa ata gag atc gga	624
His Ile Glu Gln Gly Pro Ile Leu Glu Gln Glu Gln Ile Glu Ile Gly	
195 200 205	
gtt gta acc tcc atc gtt ggc gtt cgc gca ttg cgg gtt gcc gtc aaa	672
Val Val Thr Ser Ile Val Gly Val Arg Ala Leu Arg Val Ala Val Lys	
210 215 220	

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ggc aga agc gac cac gcc ggc aca acc ccc atg cac ctg cgc cag gat 720
Gly Arg Ser Asp His Ala Gly Thr Thr Pro Met His Leu Arg Gln Asp
225 230 235 240

gcg ctg gta ccc gcc gct ctc atg gtg agg gag gtc aac cgg ttc gtc 768
Ala Leu Val Pro Ala Ala Leu Met Val Arg Glu Val Asn Arg Phe Val
245 250 255

aac gag atc gcc gat ggc aca gtg gct acc gtt ggc cac ctc aca gtg 816
Asn Glu Ile Ala Asp Gly Thr Val Ala Thr Val Gly His Leu Thr Val
260 265 270

gcc ccc ggt gga ggc aac cag gtc ccg ggg gag gtg gac ttc aca ctg 864
Ala Pro Gly Gly Gly Asn Gln Val Pro Gly Glu Val Asp Phe Thr Leu
275 280 285

gac ctg cgt tct ccg cat gag gag tcg ctc cgc gtg ctg atc gac cgc 912
Asp Leu Arg Ser Pro His Glu Glu Ser Leu Arg Val Leu Ile Asp Arg
290 295 300

atc tcg gtc atg gtc ggc gag gtc gcc tcc cag gcc ggt gtg gct gcc 960
Ile Ser Val Met Val Gly Glu Val Ala Ser Gln Ala Gly Val Ala Ala
305 310 315 320

gat gtg gat gaa ttt ttc aat ctc agc ccg gtg cag ctg gct cct acc 1008
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325 330 335

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Met Val Asp Ala Val Arg Glu Ala Ala Ser Ala Leu Gln Phe Thr His
340 345 350

cgg gat atc agc agt ggg gcg ggc cac gac tcg atg ttc atc gcc cag 1104
Arg Asp Ile Ser Ser Gly Ala Gly His Asp Ser Met Phe Ile Ala Gln
355 360 365

gtc acg gac gtc gga atg gtt ttc gtt cca agc cgt gct ggc cgg agc 1152
Val Thr Asp Val Gly Met Val Phe Val Pro Ser Arg Ala Gly Arg Ser
370 375 380

cac gtt ccc gaa gaa tgg acc gat ttc gat gac ctt cgc aaa gga act 1200
His Val Pro Glu Glu Trp Thr Asp Phe Asp Asp Leu Arg Lys Gly Thr
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<212> PRT

<213> *Arthrobacter aurescens*

<400> 5

Val Thr Leu Gln Lys Ala Gln Ala Ala Arg Ile Glu Lys Glu Ile Arg
1 5 10 15

Glu Leu Ser Arg Phe Ser Ala Glu Gly Pro Gly Val Thr Arg Leu Thr
20 25 30

Tyr Thr Pro Glu His Ala Ala Ala Arg Glu Thr Leu Ile Ala Ala Met
35 40 45

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Lys Ala Ala Ala Leu Ser Val Arg Glu Asp Ala Leu Gly Asn Ile Ile
50 55 60
Gly Arg Arg Glu Gly Thr Asp Pro Glu Leu Pro Ala Ile Ala Val Gly
65 70 75 80
Ser His Phe Asp Ser Val Arg Asn Gly Gly Met Phe Asp Gly Thr Ala
85 90 95
Gly Val Val Cys Ala Leu Glu Ala Ala Arg Val Met Leu Glu Asn Gly
100 105 110
Tyr Val Asn Arg His Pro Phe Glu Phe Ile Ala Ile Val Glu Glu Glu
115 120 125
Gly Ala Arg Phe Ser Ser Gly Met Leu Gly Gly Arg Ala Ile Ala Gly
130 135 140
Leu Val Ala Asp Arg Glu Leu Asp Ser Leu Val Asp Glu Asp Gly Val
145 150 155 160
Ser Val Arg Gln Ala Ala Thr Ala Phe Gly Leu Lys Pro Gly Glu Leu
165 170 175
Gln Ala Ala Ala Arg Ser Ala Ala Asp Leu Arg Ala Phe Ile Glu Leu
180 185 190
His Ile Glu Gln Gly Pro Ile Leu Glu Gln Glu Gln Ile Glu Ile Gly
195 200 205
Val Val Thr Ser Ile Val Gly Val Arg Ala Leu Arg Val Ala Val Lys
210 215 220
Gly Arg Ser Asp His Ala Gly Thr Thr Pro Met His Leu Arg Gln Asp
225 230 235 240
Ala Leu Val Pro Ala Ala Leu Met Val Arg Glu Val Asn Arg Phe Val
245 250 255
Asn Glu Ile Ala Asp Gly Thr Val Ala Thr Val Gly His Leu Thr Val
260 265 270
Ala Pro Gly Gly Gly Asn Gln Val Pro Gly Glu Val Asp Phe Thr Leu
275 280 285
Asp Leu Arg Ser Pro His Glu Glu Ser Leu Arg Val Leu Ile Asp Arg
290 295 300
Ile Ser Val Met Val Gly Glu Val Ala Ser Gln Ala Gly Val Ala Ala
305 310 315 320
Asp Val Asp Glu Phe Phe Asn Leu Ser Pro Val Gln Leu Ala Pro Thr
325 330 335
Met Val Asp Ala Val Arg Glu Ala Ala Ser Ala Leu Gln Phe Thr His
340 345 350
Arg Asp Ile Ser Ser Gly Ala Gly His Asp Ser Met Phe Ile Ala Gln
355 360 365
Val Thr Asp Val Gly Met Val Phe Val Pro Ser Arg Ala Gly Arg Ser
370 375 380

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His Val Pro Glu Glu Trp Thr Asp Phe Asp Asp Leu Arg Lys Gly Thr
385 390 395 400

Glu Val Val Leu Arg Val Met Lys Ala Leu Asp Arg
405 410

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Patent claims

1. rec-L-N-Carbamoylase from *Arthrobacter aurescens* and mutants thereof.
2. Amino acid sequence of the carbamoylases according to
5 claim 1.
3. Gene sequences which code the amino acid sequences according to claim 2.
4. Vectors containing the gene sequences according to claim 3.
- 10 5. Plasmids containing the gene sequences according to claim 3.
6. Guest cells containing the gene sequences according to claim 3.
7. Process for the preparation of L-amino acids by means
15 of carbamoylases according to claim 1
8. Process according to claim 7,
characterized in that
N-carbamoyl- or N-formylamino acids are reacted.
9. Process according to claim 8,
20 characterized in that
the N-carbamoylamino acids are prepared from the
corresponding hydantoins by means of hydantoinases.
10. Process according to claim 9,
characterized in that
25 the hydantoins employed are constantly racemized by
enzymatic or chemical processes.

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11. Process according to claim 7,
characterized in that
it is carried out in an enzyme membrane reactor.
12. Process according to claim 7, 8 and 9,
5 characterized in that
the enzymes employed are immobilized on carriers.
13. Process according to claim 12,
characterized in that
the rec-L-N-carbamoylase is immobilized covalently on
10 EAH-Sepharose.