

Derivatives of hirudin and the genetic engineering preparation thereof are disclosed in the European Patent Application with the publication number (EP-A) 0,171,024.

5 It has now been found that the hirudin derivative with the amino acid sequence

0 1 10
Leu-Thr-Tyr-Thr-Asp-Cys-Thr-Glu-Ser-Gly-Gln-Asn-Leu-
20
Cys-Leu-Cys-Glu-Gly-Ser-Asn-Val-Cys-Gly-Gln-Gly-Asn-
10 30
Lys-Cys-Ile-Leu-Gly-Ser-Asp-Gly-Glu-Lys-Asn-Gln-Cys-
40 50
Val-Thr-Gly-Glu-Gly-Thr-Pro-Lys-Pro-Gln-Ser-His-Asn-
60
15 Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
has a number of advantages. The numbering used in
EP-A 0,171,024 has been retained in this sequence.

20 Hirudin and its derivatives differ in biological activity, which can be attributed to differences in the affinity for thrombin and/or differences in the stability. The hirudin derivative according to the invention is, surprisingly, distinguished by special activity.

25 It has also been found that the hirudin derivative according to the invention is particularly advantageously expressed in yeasts. Comparison experiments showed that analogous hirudin derivatives starting with N-terminal Thr-Tyr or Ile-Tyr are expressed only in low yields.

30 Expression from yeast cells is advantageous not only because the hirudin derivative is secreted but also, and especially, because it is virtually quantitatively in the correctly folded form and has high activity.

Figure 1 shows cloning vectors for obtaining a gene structure which codes for the yeast MF α precursor protein and the hirudin derivative according to the invention.

Figure 2 shows a yeast expression vector having this gene structure.

5 The hirudin derivative according to the invention can, of course, also be prepared by different methods, for example by expression in bacteria or in higher eukaryotic cells such as insect cells or animal cells. However, expression from yeast systems is preferred, for example using the yeast species listed in EP-A 0,248,227, for example, *Pichia pastoris*, *Hansenula polymorphis*, 10 *Schizosaccharomyces pombe* or, preferably, *Saccharomyces cerevisiae*.

A large number of vectors are known for expression in yeasts, for example from EP-A 0,060,057, 0,088,632, 0,116,201, 0,121,884, 0,123,544 and 0,195,691. The 15 preparation of the hirudin derivative according to the invention is described hereinafter using the yeast α factor system, but this is to be understood to be merely by way of example, since other expression systems can also be used in a manner known per se.

20 The structure of the yeast pheromone gene MF α is known from Kurjan and Herskovitz, Cell 30 (1982) 933-943, where the possibility of expression of other genes and the secretion of the gene products is also discussed. In this connection, reference may also be made to Brake et al., 25 Proc. Natl. Acad. Sci. USA 81 (1984), 4642-4646.

The yeast vectors which are advantageously used as so-called shuttle vectors which have an origin of replication of a bacterial plasmid and of a yeast plasmid, as well as genes for selection in both host systems. 30 Furthermore, vectors of this type contain the promoter sequences necessary for the expression of foreign genes and, where appropriate, a terminator sequence for improving the yield, so that the heterologous gene - expediently fused to secretory signals - is located 35 between the promoter and terminator.

The invention is explained in detail by the Examples which follow. Percentage data relate to weight.

Example 1: Construction of the expression vector

5 Firstly the DNA sequence I (Table 1) is synthesized by the phosphite method. This DNA sequence codes for amino acids 49 to 80 of the MF α precursor protein and essentially corresponds to the natural DNA sequence.

10 DNA sequence I is initially used as a probe for isolating the gene for the α factor, and for this purpose is labelled with ^{32}P . This probe is used to isolate the gene from a genomic λ gt11 yeast gene bank (as are now commercially available from, for example, Clontech Laboratories Inc., 4055 Fabian Way, Palo Alto, CA94303). For this purpose, λ gt11 phages which carry the α factor
15 gene are identified in a plaque-hybridization experiment. Phages from plaques identified as positive are isolated and propagated, and the DNA is obtained. The latter is cleaved with EcoRI and analyzed on a 0.8% agarose gel. After a Southern transfer experiment, the membrane is
20 hybridized with the ^{32}P -labeled DNA sequence I. Phage DNA which has an approximately 1.75 kb fragment which hybridizes with DNA sequence I is again cleaved with the enzyme, and the corresponding fragment is isolated. The vector pUC 19 is opened with EcoRI and reacted with the
25 1.75 kb fragment using T4 ligase. The cloning vector 1 is obtained.

30 The cloning vectors which are listed in Table 2 were all constructed on the basis of a pUC plasmid. This table shows only the polylinker region of these vectors in the usual 5'-3' direction, with the MF α sequences being indicated by dotted lines, and the hirudin sequences being indicated by broken lines. Full lines denote pUC and linker sequences. Figure 1 shows these cloning vectors diagrammatically and not to scale.

The strain *E. coli* 79/02 is transformed with the ligation mixture. White colonies are isolated, the plasmid DNA is obtained from them, and plasmids which contain the 1.75 kb *EcoRI* fragment are identified.

5 The natural DNA sequence of the precursor protein for MF α contains in the region of the codons for amino acids 8 to 10 a *PstI* cleavage site and in the region of the codons for amino acids 48/49 a *TaqI* cleavage site. The fragment which codes for amino acids 9 to 48 of the MF α precursor
10 sequence is now isolated from the isolated plasmid DNA by reaction with *PstI* and *TaqI*. The vector pUC18 is opened with *PstI* and *KpnI* and is reacted with the *PstI*-*TaqI* fragment as well as with the synthetic DNA sequence I using T4 ligase. *E. coli* 79/02 is transformed with the
15 ligation mixture. The transformation mixture is plated out on IPTG-Xgal-Ap plates. White colonies are isolated, and the plasmid DNA of these clones is characterized by restriction analysis. In this way is obtained the cloning vector 2 which codes for amino acids 8 to 80 of the MF α
20 precursor sequence.

The said coding sequence is cut out of the cloning vector 2 by reaction with *PstI* and *KpnI*, and is introduced into the ligation described below. For this purpose, the cloning vector 1 is reacted with *EcoRI* and partially with
25 *PstI*, and the fragment comprising the coding sequence for the first 8 amino acids of the MF α precursor sequence is isolated. In addition, the vector pUC19 is opened with *EcoRI* and *KpnI* and ligated with the two fragments described, resulting in the cloning vector 3. The latter
30 codes for the complete MF α precursor sequence up to amino acid 80.

The starting material used for most of the hirudin sequence is the synthetic gene which is depicted in EP-A 0,171,024 as "DNA sequence I" and is shown in the present
35 Table 1 as DNA sequence IV. The restriction enzyme cleavage sites in this sequence are emphasized by

underlining: AccI cuts in the region of amino acids 1 to 3, BamHI cuts in the region of amino acids 30/31, and SacI cuts starting with the last stop codon. The protruding sequence for XbaI is located at the 5' end of the gene, and the protruding sequence for SalI is located at the 3' end.

This synthetic gene was subcloned in two parts (Figures 1 and 2 in EP-A 0,171,024). These subcloning vectors are depicted in Table 2 under No. 4 (corresponding to Figure 2 of EP-A 0,171,024) and 6 (corresponding to Figure 1 of EP-A 0,171,024).

The cloning vector 4 is opened with HincII and HindIII, and the linearized DNA is ligated with DNA sequence II (Table 1). An NcoI cleavage site has been formed at the site which has undergone blunt-ended ligation in the cloning vector 5 obtained in this way.

The fragment coding for the hirudin part-sequence is cut out of the cloning vector 6 by total digestion with BamHI and AccI. This fragment is then ligated with the cloning vector 3 which has been opened with BamHI and KpnI, and with DNA sequence III (Table 1). The last three codons in DNA sequence III are numbered in the same way as in DNA sequence IV (Table 1). This results in the cloning vector 7 which codes for the first 80 amino acids of the MFA precursor sequence and the first 30 amino acids of the hirudin derivative according to the invention, as has been confirmed by DNA sequence analysis.

The fragment which codes for amino acids 31 to 64 of hirudin is cut out of the cloning vector 5 with BamHI and HindIII. This fragment is ligated into the cloning vector 7 which has been opened with the same enzymes, resulting in the cloning vector 8 which codes for the first 80 amino acids of the MFA precursor sequence and the complete sequence of the hirudin derivative according to the invention. The structure of this plasmid is confirmed

by restriction analysis.

The plasmid Yep13 (Broach et al., Gene 8 (1979) 121) is opened with BamHI, and the protruding ends are filled in with Klenow polymerase. The DNA is precipitated with ethanol and treated with bovine alkaline phosphatase.

The fragment coding for the hirudin derivative and the MF α precursor sequence is cut out of the cloning vector 8 (Table 2) with NcoI and EcoRI, and the protruding ends are filled in as described.

10 The two blunt-ended DNA sequences are ligated together, resulting in plasmids pafHir17 and pafHir18 (Figure 1). These two plasmids differ only in the orientation of the inserted fragment.

15 It is possible to insert, as described in EP-A 0,171,024, a terminator downstream of the inserted sequence (Figures 4 to 6 of EP-A 0,171,024). Suitable for this purpose are the NcoI and/or BamHI cleavage sites.

20 After amplification of the plasmid DNA in E. coli MM294, the plasmid pafHir17 is transformed into the leucine-dependent yeast strains γ 79 (α , trp1-1, leu2-1) (Cantrell et al., Proc. Acad. Natl. Sci. USA 82 (1985) 6250) and DM6-6 (α/α leu2-3, 112::ura3⁺/leu2::lys2⁺, trp1⁻/trp1⁻, his3-11, 15/his3-11, 15, ura3⁻/ura3⁻, lys2⁻/lys2⁻, arg4-17/arg4⁺, adel⁻/adel⁺) (Maya Hanna, Dept. Mol. Biol. 25 Massachusetts General Hospital, Boston, USA) by the lithium method of Ito, H. et al., J. Bacteriol. 153 (1983) 163. Isolation of single colonies which are able to grow on selective medium without added leucine is carried out. Yeast minimal medium is inoculated with the 30 individual colonies and incubated at 28°C for 24 hours. The cells are spun down and the supernatant is examined in a thrombin inhibition assay for hirudin activity. The plasmid DNA from yeast clones whose supernatant shows hirudin activity is reisolated and characterized by

restriction analysis. The transformed yeast strains are used for the expression tests which follow.

Example 2: Expression

10 ml of yeast complete medium is inoculated with cells
5 taken from a fresh overnight culture of a strain obtained
as in Example 1, from selective medium, in such a way
that an optical density $OD_{600} = 0.1$ is reached. The
culture is shaken at 28°C for 8 hours and then 50 ml of
fresh medium are added. The culture is then shaken for a
10 further 20 hours. The cells are spun down, and the
hirudin activity in the supernatant is determined.

Example 3: Working up

Supernatant obtained as in Example 2 is acidified to pH
3 to 5 and applied to an adsorption column containing a
15 porous adsorber resin composed of a copolymer of styrene
and divinylbenzene (®DIAION HP 20) which has been
equilibrated with 0.1 M acetic acid. Washing with
Tris · HCl (pH 8.5) and 50 mM acetic acid is followed by
elution with 30% strength isopropanol. The fractions
20 containing the hirudin derivative are combined and
purified on a Q-SEPHAROSE® column which has been
equilibrated with 20 mM piperazine · HCl (pH 6). Elution
in this case is with a 0 - 0.25 M NaCl gradient. The
fractions containing the hirudin derivative are again
25 combined and purified by HPLC on a C18 reversed phase
chromatography column. The pure product obtained in this
way is then subjected to automated protein sequence
analysis.

Example 4: Comparison example

30 If the procedure of Example 1 is used but with the
following sequences in place of DNA sequence III (Table
1), then only minimal hirudin activity is detectable in
the supernatant of the yeast culture.

								1	2
		(Pro)	Leu	Asp	Lys	Arg	Thr	(Tyr)	
	5'	CT	TTG	GAT	AAA	AGA	ACG	T	
IIIIa	3'	CAT	GGA	AAC	CTA	TTT	TCT	TGC	ATA
		(KpnI)						(AccI)	

								1	2
		(Pro)	Leu	Asp	Lys	Arg	Ile	(Tyr)	
	5'	CT	TTG	GAT	AAA	AGA	ATA	T	
IIIIb	3'	CAT	GGA	AAC	CTA	TTT	TCT	TAT	ATA
		(KpnI)							

When DNA sequence IIIIb is used, the vectors corresponding to cloning vectors 7 and 8 (Table 2) do not contain the AccI cleavage site.

Table 1: DNA sequences

I. 5' C GAT GTT GCT GTT TTG CCA TTC TCC
 3' TA CAA CGA CAA AAC GGT AAG AGG
 (TaqI)

60 65

AAC AGT ACT AAT AAC GGT TTA TTG TTC

TTG TCA TGA TTA TTG CCA AAT AAC AAG

70

ATT AAT ACT ACT ATT GCT AGC ATT GCT
TAA TTA TGA TGA TAA CGA TCG TAA CGA

75		80	
GCT	AAA	GAA	GAA GGG GTA C 3'
CGA	TTT	CTT	CTT CCC 5'

(KpnI)

II. 5' CATGGA 3'
3' GTACCTTCGA 5'
(HindIII)

		(Pro)	Leu	Asp	Lys	Arg	Leu	Thr (Tyr)	
III. 5'		CT	TTG	GAT	AAA	AGA	CTT	ACG T	3'
	3'	CAT	GGA	AAC	CTA	TTT	TCT	GAA TGC ATA	5'
		(KpnI)						(AccI)	

DNA sequence IV

Triplet No.			1		0	1	2	3	4	5
Amino acid					Met	Thr	Tyr	Thr	Asp	Cys
Nucleotide No.			1			10			20	
5	Cod. strand		5'	CT	AGA	ATG	ACG	TAT	ACT	TCC
	Non-cod. strand		3'		T	TAC	TGC	ATA	TGA	ACG
6	7	8	9	10	11	12	13	14	15	
Thr	Glu	Ser	Gly	Gln	Asn	Leu	Cys	Leu	Cys	
		30			40			50		
ACT	GAA	TCT	GGT	CAG	AAC	CTG	TGC	CTG	TGC	
TGA	CTT	AGA	CCA	GTC	TTG	GAC	ACG	GAC	ACG	
16	17	18	19	20	21	22	23	24	25	
Glu	Gly	Ser	Asn	Val	Cys	Gly	Gln	Gly	Asn	
		60			70			80		
GAA	GGA	TCT	AAC	GTT	TGC	GGC	CAG	GGT	AAC	
CTT	CCT	AGA	TTG	CAA	ACG	CCG	GTC	CCA	TTG	
26	27	28	29	30	31	32	33	34	35	
Lys	Cys	Ile	Leu	Gly	Ser	Asp	Gly	Glu	Lys	
		90			100			110		
AAA	TGC	ATC	CTT	GGA	TCC	GAC	GGT	GAA	AAG	
TTT	ACG	TAG	GAA	CCT	AGG	CTG	CCA	CTT	TTC	
36	37	38	39	40	41	42	43	44	45	
Asn	Gln	Cys	Val	Thr	Gly	Glu	Gly	Thr	Pro	
		120			130			140		
AAC	CAG	TGC	GTT	ACT	GGC	GAA	GGT	ACC	CCG	
TTG	GTC	ACG	CAA	TGA	CCG	CTT	CCA	TGG	GGC	
46	47	48	49	50	51	52	53	54	55	
Lys	Pro	Gln	Ser	His	Asn	Asp	Gly	Asp	Phe	
		150			160			170		
AAA	CCG	CAG	TCT	CAT	AAC	GAC	GGC	GAC	TTC	
TTT	GGC	GTC	AGA	GTA	TTG	CTG	CCG	CTG	AAG	
56	57	58	59	60	61	62	63	64		
Glu	Glu	Ile	Pro	Glu	Glu	Tyr	Leu	Gln	Stp	
		180			190			200		
GAA	GAG	ATC	CCT	GAG	GAA	TAC	CTT	CAG	TAA	
CTT	CTC	TAG	GGA	CTC	CTT	ATG	GAA	GTC	ATT	
Stp										
		210								
TAG	AGC	TCG			3'					
ATC	TCG	AGC	AGC	T	5'					

Table 2: Cloning vectors

Nr.	pUC	
1	19	-E... (1,75 kb α -Fragment) ...E-
2	18	-K... (α -80-49) ...T... (α -48-8) ...P-
3	19	-B-K... (α -80-49) ...T... (α -48-8) ...P...E-
4	8	-B--- (Hir31-64)-S-Hc-Hd-
5	8	-B--- (Hir31-64)-S-N-Hd-
6	12	-B--- (Hir30-3)---A---X-A-
7	19	-Hd-B--- (Hir30-3)---A---K... (α -80-8) ...P...E-
8	19	-Hd-N-S--- (Hir64-3)---A---K... (α -80-8) ...P...E-

... MF α sequences

... hirudin sequences

Abbreviations for restriction enzymes

A	=	AccI
B	=	BamHI
E	=	EcoRI
Hc	=	HincII
Hd	=	HindIII
K	=	KpnI
N	=	NcoI
P	=	PstI
S	=	SalI
T	=	TaqI
X	=	XbaI



PATENT CLAIMS for the Contracting States AT, BE, CH, DE, FR,
GB, IT, LI, LU, NL, SE

1. A hirudin derivative with the amino acid sequence
0 1 10
5 Leu-Thr-Tyr-Thr-Asp-Cys-Thr-Glu-Ser-Gly-Gln-Asn-Leu-
20
Cys-Leu-Cys-Glu-Gly-Ser-Asn-Val-Cys-Gly-Gln-Gly-Asn-
30
Lys-Cys-Ile-Leu-Gly-Ser-Asp-Gly-Glu-Lys-Asn-Gln-Cys-
10 40 50
Val-Thr-Gly-Glu-Gly-Thr-Pro-Lys-Pro-Gln-Ser-His-Asn-
60
Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.
2. DNA coding for the polypeptide having the amino acid
15 sequence as claimed in claim 1.
3. Vectors containing a DNA sequence as claimed in
claim 2.
4. A process for the preparation of a polypeptide as
20 claimed in claim 1, which comprises expression of a
DNA as claimed in claim 2 in a host cell.
5. The process as claimed in claim 4, wherein the host
cell is a yeast cell.
6. A pharmaceutical containing a polypeptide as claimed
in claim 1.