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(54) Title: MUTANT α-AMYLASE, DETERGENT, DISH WASHING AGENT, AND LIQUEFACTION AGENT

(57) Abstract

In the mutant α-amylase one or more of the methionine amino acid residues is exchanged with any amino acid residue except for Cys and Met. The thus produced mutant α-amylase exhibits an improved stability in the presence of oxidizing agents and an improved thermoactivity at moderate low pH values. The mutant α-a amylase is well suited as an additive to detergents, dish washing agents and liquefaction agents.

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MUTANT α-AMYLASE, DETERGENT, DISH WASHING AGENT, AND LIQUEFACTION AGENT

The invention comprises a mutant α-amylase, a detergent, a dish washing agent, and a liquefaction agent.

Mutant amylases with improved oxidation stability, wherein one or more methionines have been mutated into cysteins or chemically modified cysteins are described in WO 91/16423. The reason for research in this field is the need for oxidation stable α-amylases as additives for detergents and dish washing agents, due to the presence of strong oxidizing agents in the detergents and the dish washing agents, as explained in more detail in WO 91/16423.

It has been found that the activity level and the stability in the presence of oxidizing agents of the prior art mutant amylases is open to improvement, and thus, the purpose of the invention is the provision of a mutant α-amylase with an improved activity level and an improved stability in the presence of oxidizing agents in comparison to the prior art mutant amylases. In this context the term "stability in the presence of oxidizing agents" refers both to the storage stability of the α-amylase during storage of the α-amylase product and during storage of the α-amylase containing detergent or the α-amylase containing dish washing agent, and to the stability in the washing solution or dish washing solution during the washing process or dish washing process, and furthermore to the stability of the α-amylase during hydrolysis of starch in the presence of hydrogen peroxide or other bleaching agents, e.g. during desizing in the textile industry.

The mutant α-amylase according to the invention is characterized by the fact that one or more of the methionine amino acid residues is exhanged with any amino acid residue except for Cys and Met. Thus, according to the invention the amino acid residues to replace the methionine amino acid residue are the following: Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

Surprisingly it has been found that the mutant α-amylase according to the invention exhibits a better activity level and a better stability in the presence of oxidizing agents than the prior art mutant amylases.

A preferred embodiment of the mutant α-amylase according to the invention is characterized by the fact that the α-amylase is a *Bacillus* α-amylase. *Bacillus* α-amylase exhibit in themselves a high heat stability, and by being mutated according to the invention the mutants exhibit an even better stability, especially in the presence of oxidizing agents.

A preferred embodiment of the mutant α-amylase according to the invention is characterized by the fact that the α-amylase is *B. licheniformis* α-amylase, *B. amyloliquefaciens* α-amylase, *B. stearothermophilus* α-amylase, *Asp. oryzae* α-amylase, or *Asp. niger* α-amylase. These α-amylases are all well characterized and their entire amino acid sequence is described. It is to be understood that an α-amylase, which is identical to the *B. licheniformis* α-amylase identified in FR 2665178, except for the fact that it exhibits an arginin residue in position 23 instead of the lysin residue, also belongs to the α-amylases, which are basis for the mutant α-amylases according to the invention.

A preferred embodiment of the mutant α-amylase according to the invention is characterized by the fact that the α-amylase exhibits an amino acid sequence with a homology of at least 60% in relation to the following mutant α-amylases: *Bacillus* α-amylase, preferably *B. licheniformis* α-amylase, *B. amyloliquefaciens* α-amylase, and *B. stearothermophilus* α-amylase, and furthermore *Aspergillus niger* α-amylase. It has been found that this entire group of mutant α-amylases exhibit a satisfactory stability in the presence of oxidizing agents.

A preferred embodiment of the mutant α-amylase according to the invention is characterized by the fact that one or more of the methionine amino acid residues is (are) exchanged with a Leu, Thr, Ala, Gly, Ser, Ile, or Asp amino acid residue, preferably a Leu, Thr, Ala, or Gly amino acid residue. In this embodiment a very satisfactory activity level and stability in the presence of oxidizing agents is obtained.

A preferred embodiment of the mutant α-amylase according to the invention is characterized by the fact that the methionine amino acid residue in position 197 in *B. licheniformis* α-amylase or the methionine amino acid residue in homologous positions in other α-amylases is exchanged. The concept of homologous positions or sequence homology of α-amylases has been explained

e.g. in Nakajima, R. et al., 1986, Appl. Microbiol. Biotechnol. 23, 355-360 and Liisa Holm et al., 1990, Protein Engineering 3, 181-191. Sequence homology of *Bacillus* α-amylases from *B. licheniformis*, *B. stearothermophilus* and *B. amyloliquefaciens* are about 60%. This makes it possible to align the sequences in order to compare residues at homologous positions in the sequence. By such alignment of α-amylase sequences the number in each α-amylase sequence of the homologous residues can be found. The homologous positions will probably spatially be in the same position in a three dimensional structure (Greer, J., 1981, J. Mol. Biol. 153, 1027-1042), thus having analogous impact on specific functions of the enzyme in question. In relation to position 197 in *B. licheniformis* α-amylase the homologous positions in *B. stearothermophilus* α-amylase are positions 200 and 206, and the homologous position in *B. amyloliquefaciens* α-amylase is position 197. Experimentally it has been found that these mutants exhibit both an improved activity level and an improved stability in the presence of oxidizing agents.

A preferred embodiment of the mutant α-amylase according to the invention is characterized by the fact that one or both of the methionine amino acid residues in positions 200 and 206 in *B. stearothermophilus* α-amylase or the methionine amino acid residues in homologous positions in other α-amylases are exchanged. In relation to positions 200 and 206 in *B. stearothermophilus* α-amylase the homologous position in *B. licheniformis* α-amylase is 197 and the homologous position in *B. amyloliquefaciens* α-amylase is position 197. Experimentally it has been found that these mutants exhibit both an improved activity level and an improved stability in the presence of oxidizing agents.

Also, the invention comprises a detergent, which is characterized by the fact that it comprises the mutant α-amylase according to the invention. Thus, according to the the invention, the mutant α-amylase may be added as a component of a detergent composition. As such, it may be included in the detergent composition in the form of a detergent additive. The detergent composition as well as the detergent additive may additionally comprise one or more other enzymes conventionally used in detergents, such as proteases, lipases, cellulases, oxidases or peroxidases.

In a specific aspect, the invention provides a detergent additive. The enzymes may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes.

Preferably, the detergent additive, i.e. a separated additive or a combined additive, is provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or in a protected form.

Dust free granulates may be produced, e.g. as disclosed in US 4,106,991 and US 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. The detergent enzymes may be mixed before or after granulation.

Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as e.g. propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid, according to established methods. Other enzyme stabilizers are well known in the art.

Protected enzymes may be prepared according to the method disclosed in EP 238,216 A.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules or liquid. A liquid detergent may be aqueous, typically containing up to 90% water and 0-20% organic solvent.

The detergent composition comprises a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 0-50% anionic surfactant such as linear alkyl benzene sulphonate (LAS), alpha-olefin sulphonate (AOS), alkyl sulphate (AS), alcohol ethoxy sulphate (AES) or soap. It may also contain 0-40% non-ionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate. Furthermore, it may contain a polyhydroxy fatty acid amide surfactant (e.g. as described in WO 92/06154).

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-11. The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, alkenyl succinic anhydride, or silicate, or it may be unbuilt (i.e. essentially free of a detergent builder). It may also contain other conventional detergent ingredients, e.g. fabric conditioners,

foam boosters, bleaching agents, e.g. perborate, percarbonate, tetraacetyl ethylene diamine (TAED), or nonanoyloxybenzene sulfonate (NOBS), anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, stabilizing agents for the enzyme(s), foam depressors, dyes, bactericides, optical brighteners or perfumes.

Particular forms of detergent compositions within the scope of the invention include:

a) A detergent composition formulated as a detergent powder containing phosphate builder, anionic surfactant, nonionic surfactant, silicate, alkali to adjust to desired pH in use, and neutral inorganic salt.

b) A detergent composition formulated as a detergent powder containing zeolite builder, anionic surfactant, nonionic surfactant, acrylic or equivalent polymer, silicate, alkali to adjust to desired pH in use, and neutral inorganic salt.

c) A detergent composition formulated as an aqueous detergent liquid comprising anionic surfactant, nonionic surfactant, humectant, organic acid, alkali, with a pH in use adjusted to a value between 7 and 10.5.

d) A detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant consisting essentially of linear alkoxylated primary alcohol, phosphate builder, alkali, with a pH in use adjusted to a value between about 7 and 10.5.

e) A detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and nonionic surfactant, low or substantially zero neutral inorganic salt, phosphate builder, and sodium silicate.

f) A detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and nonionic surfactant, low or substantially zero neutral inorganic salt, zeolite builder, and sodium silicate.

g) A detergent composition formulated as a detergent powder containing anionic surfactant, nonionic surfactant, acrylic polymer, fatty acid soap, sodium carbonate, sodium sulphate, clay particles, and sodium silicate.

h) A liquid compact detergent comprising 5-65% by weight of surfactant, 0-50% by weight of builder and 0-30% by weight of electrolyte.

It is at present contemplated that, in the detergent composition of the invention, the mutant α-amylase may be added in an amount corresponding to 0.001-100 mg of enzyme per liter of wash liquor.

Also, the invention comprises a dish washing agent, which is characterized by the fact that it comprises the mutant α-amylase according to the invention. All dish washing agent formulations can be used in combination with the mutant α-amylase according to the invention.

Both the detergent according to the invention and the dish washing agent according to the invention may comprise oxidizing agents, which may be an activator, a bleaching agent or an oxidizing enzyme. The bleaching agent may be a chlorine containing agent, preferably a hypochlorit generating agent, a percarbonate, or a perborate.

Also, the invention comprises a liquefaction agent, which is characterized by the fact that it comprises the mutant α-amylase according to the invention. Surprisingly, it has been found that the mutant α-amylase according to the invention besides the improved stability in the presence of oxidizing agent also possesses an improved thermoactivity at moderate low pH values, which is highly advantageous for liquefaction agents. Documentation for this improved thermoactivity will be presented later.

From the Journal of Biological Chemistry, Vol. 260, No. 11, pages 6518-6521 it appears that site-directed mutagenesis can be employed to alter critical residues in proteins which are susceptible to chemical oxidation, and that methionine 222 is a primary site for oxidative inactivation of subtilisin. This citation, however, does not describe mutants of α-amylase, whereas the invention is strictly limited to mutants of α-amylase.

The amino acid sequence for the *B. licheniformis* α-amylase appears from J. Bacteriol. 166, 635-643, 1986, FR 2665178 or EP 410498. Thus, the methionine numbers are: 8, 15, 197, 256, 304, 366, and 438.

The amino acid sequence for the *B. amyloliquefaciens* α-amylase appears from J. Biol. Chem. 258, 1007-1013, 1983. Thus, the methionine numbers are: 6, 197, 256, 304, 366, and 438.

The amino acid sequence for the *B. stearothermophilus* α-amylase appears from J. Bacteriol. 166, 635-643, 1986. Thus, the methionine numbers are: 8, 9, 97, 200, 206, 284, 307, 311, 316, and 437.

The amino acid sequence for the *Asp. oryzae* α-amylase (sold commercially as FUNGAMYL®, by Novo Nordisk A/S) as is follows:

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  1 ATPADWRSQS IYFLLTDRFA RTDGSTTATC
 31 NTADQKYCGG TWQGIIDKLD YIQGMGFTAI
 61 WITPVTAQLP QTTAYGDAYH GYWQQDIYSL
 91 NENYGTADDL KALSSALHER GMYLMVDVVA
121 NHMGYDGAGS SVDYSVFKPF SSQDYFHPFC
151 FIQNYEDQTQ VEDCWLGDNT VSLPDLDTTK
181 DVVKNEWYDW VGSLVSNYSI DGLRIDTVKH
211 VQKDFWPGYN KAAGVYCIGE VLDGDPAYTC
241 PYQNVMDGVL NYPIYYPLLN AFKSTSGSMD
271 DLYNMINTVK SDCPDSTLLG TFVENHDNPR
301 FASYTNDIAL AKNVAAFIIL NDGIPIIYAG
331 QEQHYAGGND PANREATWLS GYPTDSELYK
361 LIASANAIRN YAISKDTGFV TYKNWPIYKD
391 DITIAMRKGT DGSQIVTILS NKGASGDSYT
421 LSLSGAGYTA GQQLTEVIGC TTVTVGSDGN
451 VPVPMAGGLP RVLYPTEKLA GSKICSSS
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Composition

37 Ala A	19 Gln Q	33 Leu L	36 Ser S
10 Arg R	12 Glu E	20 Lys K	39 Thr T
26 Asn N	42 Gly G	9 Met M	10 Trp W
42 Asp D	7 His H	14 Phe F	34 Tyr Y
9 Cys C	29 Ile I	21 Pro P	29 Val V

Thus, the methionine numbers are: 55, 112, 115, 123, 246, 269, 275, 396, and 455.

The amino acid sequence for the *Asp. niger* α-amylase appears from DK patent application no. 5126/87. Thus, the methionine numbers are: 55, 112, 115, 123, 161, 275, 396, and 455.

Once an exchange pattern is decided upon the genetically engineered protein can be synthesized without any inventive effort according to prior art methods.

FR 2665178 describes a thermostable variant of the α-amylase from *B. licheniformis*. This variant, however, does not involve a methionine exchange, but an exchange in the neighbourhood of histidine 133, and also, the oxidative stability of this prior art α-amylase is inferior in comparison to the oxidative stability of the α-amylase mutant according to the invention.

Documentation for the surprising properties of the mutant α-amylase according to the invention, e.g. the improved activity level and the improved stability in the presence of oxidizing agents, will be presented in the following.

Documentation for improved stability in the presence of oxidizing agents

Raw filtered culture broths with different Termamyl® mutants indicated below are diluted to an amylase activity of 100 NU/ml (the NU amylase activity unit is defined in AF 207/1, which is available on request from Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark) in 50 mM of a Britton-Robinson buffer at pH 9.0 and incubated at 40°C. Subsequently H_2O_2 is added to a concentration of 200 mM, and the pH value is re-adjusted to 9.0. The activity is now measured after 15 seconds and after 5, 15, and 30 minutes. The results appear from the following table 1, in which the amylase mutant is identified by means of the one letter amino acid system. Thus, M197A means the Termamyl® mutant, in which the methionine in position 197 is exchanged with alanine. It clearly appears from Table 1 that the prior art Cystein mutant (M197C) exhibits a very low stability, even lower than the stability of Termamyl®.

The values in the following Table 1 is the OD_{620} absorption values, which are taken as an indication of the activity.

Table 1

	OD_{620} absorption values			
	15 seconds (0)	5 minutes	15 minutes	30 minutes
M197A	1.45	1.68	1.83	1.61
M197C	1.54	0.61	0.17	0.10
M197D	1.33	0.98	1.51	1.22
M197G	0.91	1.38	1.43	1.02
M197H	1.58	1.24	1.51	1.52
M197L	1.67	1.38	1.50	1.56
M197T	1.95	1.80	1.59	1.89
M197V	1.39	1.42	1.33	1.55
M197I	1.57	1.39	1.30	1.34
M197S	1.44	1.39	1.34	1.51
M197N	1.27	1.20	1.39	1.46
Termamyl®	1.66	1.08	0.54	0.29

Documentation for improved activity level in the presence of oxidizing agents

All mutants are purified to homogeneity, and the absorption A_{280} is taken as an indication of the protein content. The purified samples were diluted until a value of A_{280} of 0.0014, corresponding to 4 NU/ml in relation to Termamyl®. Under these circumstances the specific activity of each mutant was measured in the presence of a strong oxidizing agent (peracetic acid) and as a comparison in the absence of the strong oxidizing agent. The activities were determined as described in AF 207/1 but with a standard curve constructed at pH 9.0, 60°C instead of at pH 7.3, 37°C. Termamyl® was used as reference in the standard curve. The activity units are therefore referred to as NU*. It clearly appears from Table 2, in which the values of the specific activities are given that the activity level in the environment comprising the strong oxidizing agent in comparison to the control is superior for all mutants, when compared to Termamyl®.

Table 2

	60°C, pH 9.0	60°C, pH 9.0 5 mM peracetic acid
Termamyl®	2271 NU*	528 NU*
M197A	1743 NU*	1328 NU*
M197G	1708 NU*	1329 NU*
M197T	1535 NU*	1256 NU*
M197V	892 NU*	771 NU*
M197Q	999 NU*	802 NU*
M197F	514 NU*	421 NU*
M197L	1515 NU*	1686 NU*
M197H	792 NU*	472 NU*
M197I	1456 NU*	1276 NU*
M197S	1968 NU*	1102 NU*
M197N	1750 NU*	1556 NU*

Documentation for improved thermoactivation at moderate low pH values

Termamyl® variants M197A and M197T and Termamyl®, respectively, were purified to homogeneity, and dilution was made according to A_{280} in 50mM Britton-Robinson buffer. Dilution were made to reach A_{620} absorption values between 0.5 and 2 measured in Phasebas assay described in Af 207/1. The respective pH values were adjusted to the indicated pH values between 4 and 10.5. After measurement of the A_{620} values all results were adjusted to A_{620} units/A_{280} (enzyme content), *vide* Table 3.

The values in the following Table 3 are A_{620} absorptions, which are taken as indications of the activities.

Table 3

A_{620} Phadebas units / A_{280} (enzyme content)								
	37°C		60°C				90°C	
pH	Termamyl®	M197A	Termamyl®	M197A	Termamyl®	M197T	Termamyl®	M197A
4.0	217	113	1045	464	1045	177	0	0
5.0	876	903	3551	5185	3551	3828	9142	13272
6.0	1067	1088	3810	5673	3810	4088	8405	12566
7.0	1104	1080	3711	5081	3711	3813	6678	8936
8.0	1131	1061	3576	4997	3576	3404	3993	3965
9.0	1005	731	2129	3256	2129	1692	913	191
10.0	685	264	1937	1042	1937	369	0	0
10.5	560	164	1429	564	1429	182	0	0

Documentation for improved storage stability of the mutants as pseudo prill in the presence of detergent

The purified mutant samples were lyophilized. Detergent and 75°C hot Berol 08 was added under heavy stirring. After hardening of the wax the product is transferred to a freezer and after a couple of hours the products is crushed and mixed with detergent.

The storage was conducted in closed vials at 37°C.

The percentage residual activities after storage under the described conditions are given in the following Table 4.

Table 4

	Days			
	0	3	7	14
Termamyl®	100%	32%	16%	10%
M197L	100%	56%	30%	20%
M197T	100%	66%	36%	23%
M197Q	100%	56%	56%	20%
M197F	100%	65%	54%	34%

Detergent composition:

9% sodium perborate monohydrate
6% TAED
2% AEO
25% sodium disilicate
2.0% phosphonate
1.2% polycarboxylates
30% trisodium citrate
14.8% sodium carbonate
10% sodium sulfate

Documentation for improved stability of the mutants in detergent slurries

The samples of purified mutants were incubated with a protein concentration of 1 mg/ml in a suspension of 5% w/w of ADD (ADD is an abbreviation of Automatic Dishwashing Detergent) detergent at 30°C and pH was adjusted to 10.5. ADD contains oxidizing agent (sodium perborate) and TAED.

The reduction in activity of the different mutants was followed during 270 minutes.

The percentage residual activities during incubation are given in the following Table 5 ("0" minutes is an initial measurement after addition of the detergent suspension).

Table 5

	Minutes			
	0	"0"	60	270
Termamyl®	100%	39%	14%	8%
M197L	100%	85%	67%	61%
M197A	100%	81%	44%	34%
M197T	100%	90%	53%	42%

CLAIMS

1. Mutant α-amylase, characterized by the fact that in the mutant α-amylase one or more of the methionine amino acid residues is exchanged with any amino acid residue except for Cys and Met.

2. Mutant α-amylase according to Claim 1, characterized by the fact that the α-amylase is a *Bacillus* α-amylase.

3. Mutant α-amylase according to Claim 1 or 2, characterized by the fact that the α-amylase is *B. licheniformis* α-amylase, *B. amyloliquefaciens* α-amylase, *B. stearothermophilus* α-amylase, *Asp. oryzae* α-amylase, or *Asp. niger* α-amylase.

4. Mutant α-amylase according to Claims 1 - 3, characterized by the fact that the α-amylase exhibits an amino acid sequence with a homology of at least 60% in relation to the mutant α-amylases according to Claim 2 or 3.

5. Mutant α-amylase according to Claims 1 - 4, characterized by the fact that one or more of the methionine amino acid residues is (are) exchanged with a Leu, Thr, Ala, Gly, Ser, Ile, or Asp amino acid residue, preferably a Leu, Thr, Ala, or Gly amino acid residue.

6. Mutant α-amylase according to Claims 1 - 5, characterized by the fact that the methionine amino acid residue in position 197 in *B. licheniformis* α-amylase or the methionine amino acid residue in homologous positions in other α-amylases is exchanged.

7. Mutant α-amylase according to Claims 1 - 5, characterized by the fact that one or both of the methionine amino acid residues in positions 200 and 206 in *B. stearothermophilus* α-amylase or the methionine amino acid residues in homologous positions in other α-amylases are exchanged.

8. Detergent, characterized by the fact that it comprises the mutant α-amylase according to Claims 1 - 7.

9. Dish washing agent, characterized by the fact that it comprises a mutant α-amylase according to Claims 1 - 7.

10. Liquefaction agent, characterized by the fact that it comprises a mutant α-amylase according to Claims 1 - 7.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00230

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 9/28, C12N 9/30, C11D 3/386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The Journal of Biological Chemistry, Volume 260, No 11, June 1985, David A. Estell et al., "Engineering an Enzyme by Sitedirected Mutagenesis to Be Resistant to Chemical Oxidation", page 6518 - page 6521, see 6518 left column, line 1-6, fig 2 --	1-10
A	WO, A1, 9116423 (NOVO NORDISK A/S), 31 October 1991 (31.10.91), page 8, line 24 - line 26 --	1-10
A	WO, A1, 8907642 (GISTBROCADES), 24 August 1989 (24.08.89), claim 14 --	1-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
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Date of the actual completion of the international search	Date of mailing of the international search report
20 October 1993	26 -10- 1993
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00230

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