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D E C I S I O N
of 6 July 1999

Case Number: T 0915/94 - 3.3.4

Application Number: 84304252.4

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Language of the proceedings: EN

Title of invention:
Procaryotic carbonyl hydrolases, methods, DNA, vectors and transformed hosts for producing them, and detergent compositions containing them

Applicant/Patentee:
Genencor International, Inc.

Opponent:
Showa Denko Kabushiki Kaisha
Novo Nordisk A/S

Headword:
Carbonyl hydrolases/GENENCOR INTERNATIONAL INC.

Relevant legal provisions:
EPC Art. 123(2), 83, 56

Keyword:
"Main request sufficiency of disclosure - (yes)"
"Inventive step - yes"

Decisions cited:
T 0964/92

Catchword:

-

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Case Number: T 0915/94 - 3.3.4

D E C I S I O N
of the Technical Board of Appeal 3.3.4
of 6 July 1999

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Decision under appeal: **Interlocutory decision of the Opposition Division of the European Patent Office posted 11 October 1994 concerning maintenance of European patent**

No. 0 130 756 in amended form.

Composition of the Board:

Chairman: U. M. Kinkeldey
Members: R. E. Gramaglia
W. Moser

Summary of Facts and Submissions

I. European Patent No. 0 130 756 (application No. 84 304 252.4) was granted on the basis of 22 claims. The patent relates to procaryotic carbonyl hydrolases, methods, DNA, vectors and transformed hosts for producing them, and detergent compositions containing them.

II. Claim 1 as granted read as follows:

"1. A process which comprises effecting a mutation in a *Bacillus* subtilisin enzyme or its pre- or preproenzyme in one or more of the positions corresponding to Tyr-1, Asn+155, Tyr+104, Met+222, Gly+166, Gly+169, Glu+156, Ser+33, Phe+189, Tyr+217 and Ala+152 in *B. amyloliquefaciens* subtilisin or its pre- or preproenzyme, and testing for a desired activity change in the enzyme resulting from said mutation."

Claims 2 to 7 were directed to special embodiments of the process of claim 1. Claims 8 and 9 related to the use of the mutated enzyme in detergent compositions.

Independent claim 10 as granted read as follows:

"10. A process which comprises:

providing a DNA sequence encoding a *Bacillus* subtilisin enzyme in which there has been made a mutation in one or more of the positions corresponding to Tyr-1, Asn+155, Tyr+104, Met+222, Gly+166, Gly+169, Glu+156, Ser+33, Phe+189, Tyr+217 and Ala+152 in *B. amyloliquefaciens*

subtilisin or its pre- or preproenzyme, transforming a host cell with the DNA so that the coding sequence will be expressed therein, and testing for a desired activity change in the enzyme resulting from said mutation."

Claims 11 to 22 were directed to special embodiments of the process of claim 10.

III. Notices of opposition were filed by opponents 01 to 03 all requesting the revocation of the European patent on the grounds of Articles 100(a), (b) and (c) EPC, i.e. lack of novelty (Article 54 EPC), lack of inventive step (Article 56 EPC), insufficiency of disclosure (Article 83 EPC) and added subject-matter (Article 123(2) EPC).

IV. The opposition division applied the problem-solution approach for evaluating the inventive step and came to the conclusion that the claims of the main and first to fourth auxiliary requests did not solve any problem other than providing a different way of modifying the amino acid sequence of subtilisin in order to obtain a desired activity change. But the "general" solution proposed by claim 1 of these requests was obvious in the light of documents (5) to (7), (30), (31) and (34), showing that the residues referred in the claims were of special importance for the interaction with the substrate. The patent was, however, maintained on the basis of the claims of the fifth auxiliary request.

Claim 1 of the main request read as follows (amendments over granted claim 1 are shown in bold):

"1. A process which comprises effecting a mutation in a **DNA**

encoding a *Bacillus* subtilisin enzyme or its pre- or preproenzyme at one or more of the positions corresponding to Tyr-1, Asn+155, Tyr+104, Met+222, Gly+166, Gly+169, Glu+156, Ser+33, Phe+189, Tyr+217 and Ala+152 in *B. amyloliquefaciens* subtilisin or its pre- or preproenzyme, and testing for a desired activity change in the enzyme resulting from said mutation."

V. The following documents are referred to in the present decision:

(1) Ulmer K.M., Science, Vol. 219, pages 666-671 (1983)

(2) Winter G. et al., Nature, Vol. 299, pages 756-758 (1982)

(3) Dalbadie-McFarland G. et al., Proc. Natl. Acad. Sci. USA, Vol. 79, pages 6409-6413 (1982)

(4) Markland F.S. et al., J. Biol. Chem., Vol. 242, No. 22, pages 5198-5211 (1967)

(5) Staufer C.E. et al., J. Biol. Chem., Vol. 244, No. 19, pages 5533-5538 (1969)

(6) Schubert Wright C. et al., Nature, Vol 221, pages 235-242 (1969)

(7) Robertus J.D. et al., Biochemistry, Vol. 11, No. 23, pages 4293-4303 (1972)

(8) Kraut J. et al., Cold Spring Harbor Symp. Quant. Biol.,

Vol. 36, pages 117-123 (1971)

(9) Robertus J.D. et al., Biochemistry, Vol. 11, No. 13, pages 2439-2449 (1972)

(10) Matthews D.A. et al., J. Biol. Chem., Vol. 250, No. 18, pages 7120-7126 (1975)

(11) Poulos T.L. et al., J. Biol. Chem., Vol. 251, No. 4, pages 1097-1103 (1976)

(12) Svendsen I., Carlsberg Res. Commun., Vol. 41, No. 5, pages 238-291 (1976)

(24) Zoller M.J. et al., Methods in Enzymology, Vol. 100, pages 468-500 (1983)

(25) Wilkinson A.J. et al., Nature, Vol. 307, pages 187-188 (1984)

(30) Smith E.L. et al. in "Structure-Function Relationships of Proteolytic Enzymes", Munsgaart, Copenhagen, DK, pages 160-172 (1970)

(31) Ottesen M. et al., in "Structure-Function Relationship of Proteolytic Enzymes", Munsksgaard, Copenhagen, DK pages 175-186 (1970)

(34) Kraut J., Ann. Rev. Biochem., Vol. 46, pages 331-349 (1977)

(43) Rastetter W.H., Trends in Biotechnology, Vol. 1, No. 3,

pages 80-84 (1983)

(44) Mitsui Y. et al., Nature, Vol. 277, pages 447-452 (1979)

(49) Data on subtilisin mutations at the claimed sites submitted by appellant I before the examining division on 6 September 1986

(50) Data of substrate specificity upon mutations at the claimed sites in BPN' and other subtilisins submitted by appellant I before the opposition division on 16 March 1994

VI. Appeals were lodged by appellant I (patentee) and appellants II and III (opponents 01 and 03). After having lodged an appeal as well, the respondent (opponent 02) withdrew the appeal with letter dated 11 August 1995.

VII. On 4 May 1999, the board issued a communication pursuant to Article 11(2) of the Procedure before the Boards of Appeal expressing its provisional opinion.

VIII. Oral proceedings were held on 6 July 1999, during which appellant I submitted a new main request and new auxiliary requests 1 to 5 in replacement of any preceding requests. The claims of the main request differed from those submitted before the opposition division as main request (see section IV supra) in that claim 5 now included a reference to claim 1 or claim 2 instead of claim 4.

IX. The submissions and evidence provided in writing and during

the oral proceedings by appellant I as regards the main request can be summarized as follows:

Article 83 EPC (Sufficiency of disclosure)

- Although the patent in suit exemplified mutations in B. amyloliquefaciens subtilisin only, its teachings were broadly applicable to any subtilisin because the skilled person was able to find "equivalent" residues in other subtilisins.

- Subtilisins had strong similarities in their structures and conserved residues indicated that one could expect that a site which was suitable for mutation in one subtilisin would have been suitable for mutation in another subtilisin (eg Met+222 in B. amyloliquefaciens (BPN') subtilisin corresponded to Met+222 in B. Carlsberg subtilisin and Met+216 in B. lentis subtilisin).

Inventive step

- The closest prior art was represented by documents (1) and (43) dealing with enzyme engineering. The invention as embodied by the claims of the main request lay in the identification of 11 sites for mutations out of 275 of the mature subtilisin protein at which sites one could substitute and obtain variation in properties of the enzyme in ways that were very likely to be useful, while still retaining enzyme function.

- The skilled person would not have expected that it was

possible to engineer subtilisins by recombinant DNA technology, so as to obtain new useful properties.

- Contrary to the opposition division's conclusion, no prior art document suggested any "promising candidates" sites. The prior art documents showed a great many sites which were around or within the "binding pocket" but only few of them turned out to be suitable for modification. A prerequisite for arriving at the claimed subject-matter was to refine the 3D structure to get a more accurate picture since there was structural information on only one subtilisin.

- Chemical modification studies were not specific for a particular residue and were also non-stoichiometric. Thus, the activity observed in the modified protein could not be ascribed exclusively to one well defined modified site.

- Document (5) did not show that the position Met+222 was a promising candidate. Document (7), page 4301 told that a sulphur atom was necessary at this position.

- The general perception was that mutation was most likely to result in no change or in a catastrophic loss of activity, especially if conserved amino acids were replaced.

- The polypeptide as expressed was not the native sequence. It could thus not be predicted that it would have folded properly and would have been processed properly from the pro-sequence.

Reimbursement of the appeal fee

- Appellant I complained that the opposition division arrived at its conclusion before the oral proceedings on the basis that it was a settled issue that inventive step had to be considered for each of the 11 sites separately and appellant I was specifically prevented by the opposition division during the oral proceedings from arguing a broader case. This amounted to a violation of the right to be heard (Article 113 EPC) justifying a reimbursement of the appeal fee (Rule 67 EPC).

X. The submissions and evidence provided by appellants II and III and by the respondent as regards the main request can be summarized as follows:

Article 123(2) EPC

- Replacement of Met+222 with Ala or Ser achieved an increase in oxidation stability (see Example 17), while replacement of Met+222 with Cys achieved a modified pH-activity profile of the sharper type (see Example 19) and a slight increase in oxidation stability (see Fig. 14). Thus, insofar as claim 5 of the main request implied that replacement of Met+222 with Ala or Ser achieved a modified pH-activity profile, the requirements of Article 123(2) EPC were not met by these claims.

Article 83 EPC

- B. subtilisins were a broad class of enzymes

having different amino acid sequences and that the few isolated examples of the patent in suit relating to B. amyloliquefaciens subtilisin only did not allow a generalization of the claimed mutations to any subtilisin and to any amino acid substitution(s). Thus, the patent in suit did not disclose which mutations at which positions and in which subtilisins had to be made in order to obtain a defined activity change.

Article 56 EPC

- Documents (6) to (11), (31), (34) and (44) provided the skilled person with the crystal structure of subtilisins and identified 16 residues which were of special importance for the interaction with the substrate, namely **Ser+33**, His+67, **Tyr+104**, Ser+125, Leu+126, Gly+127, **Ala+152**, Ala+153, Gly+154, **Asn+155**, **Glu+156**, Val+165, **Gly+166**, Tyr+167, Pro+168, **Phe+189**, **Tyr+217**, **Met+222**, including 8 (shown in bold) of the 11 sites referred to in the claims. For instance, document (6) (page 240, r.h, column, 1st paragraph) disclosed that Ala+152, Asn+155, Glu+156, Tyr+217 and Met+222 were situated on the surface of the enzyme and were to be found within 10D of the active site (Ser+221). Thus these 16 residues were the sites to be modified because the skilled person knew that replacing an amino acid within or near the "binding pocket" of an enzyme would have resulted in altered properties of the enzyme.

- A series of documents dealt with chemical

modifications of both subtilisin BPN' and Carlsberg at the specific sites recited in the claims, resulting in altered catalytic parameters (documents/position/chemical modification: document (5)/Met+222/oxidation with H_2O_2; document (12), page 270 and (30), page 165/Tyr+104/ nitration or iodination; document (12), page 270/ Glu+156 and Ser+156/glutarylation or succinylation; document (12), page 268/Tyr+217/ nitration or iodination). This suggested to the skilled person that alteration of these sites through the technique of the site-directed mutagenesis known from documents (1), (2), (3), (24) and (25) would have brought about an activity change. The latter documents also showed that it was possible to select advantageous sites.

- The properties "altered substrate specificity" and "altered pH activity" were meaningless features and thus not appropriate for supporting an inventive step.

XI. Appellants II and III requested that the decision under appeal be set aside and that the European patent No. 0 130 756 be revoked.

Appellant I requested that the decision under appeal be set aside and that the patent be maintained on the basis of the following claim requests:

(a) claims 1 to 20 submitted during oral proceedings as main request; or

(b) claims 1 to 20 filed as first auxiliary request;

or

(c) claims 1 to 19 filed as second auxiliary request; or

(d) claims 1 to 20 filed as third auxiliary request; or

(e) claims 1 to 18 filed as fourth auxiliary request; or

(f) claims 1 to 12 filed as fifth auxiliary request, all auxiliary requests being filed on 10 June 1999.

Appellant I further requested reimbursement of the appeal fee.

Reasons for the Decision

1. The appeal is admissible.

Main request
Article 123 (2)(3) EPC

2. Appellants II and III argued that the requirements of Article 123(2) EPC were not met since claim 5 of the main request, owing to its dependency on claim 4, implied that replacement of Met+222 with Ala or Ser achieved a modified pH-activity. But this had no support in the application as filed. However, claim 5 now no longer depends on claim 4 (see section VI

supra). Therefore, the claims of the main request fulfil the requirements of Article 123(2) EPC. Appellants II and III never argued that the claims of the main request were broader in scope than the granted claims and the board also sees no infringement of Article 123(3) EPC.

Article 83 EPC

3. It was argued by appellant II that undue burden would be required to find "respective positions" to the selected sites of any subtilisin covered by the claims. The board, however, is of the opinion that before the priority date of the patent in suit, it was within the reach of the skilled person to identify by homology comparisons "corresponding sites" in enzymes because document (4) (see page 5211, l-h column, fourth full paragraph) states that residue 221 in subtilisin corresponds to residue 195 of chymotrypsin. These two enzymes belong to the mammalian protease family which diverged early in the course of evolution (see ibidem). Also document (7), published 1972, on page 4303, last paragraph, teaches that Ser+221, Asn+155 and Met+222 of subtilisin BPN' corresponds to Ser+195, Gly+193 and Cys+42 of á-chymotrypsin. This demonstrates that in 1967, ie the year of publication of document (4), the skilled person has already been in a position to find corresponding sites even in distant proteases, all the more so in subtilisins belonging to the **same** family.

As for the argument that the few isolated examples in the patent in suit do not allow a generalization to any amino acid substitution(s), it is the board's view that subtilisins have strong similarities in their

structures and a great many conserved residues (see eg document (12), page 240, Fig.1), indicating that one can reasonably expect that a site which is suitable for mutation in one subtilisin will also be suitable for mutation in another subtilisin. There certainly is no experimental evidence to the contrary before the board. In view of the above, it must be concluded that the claims of the main request satisfy the requirements of Article 83 EPC.

Article 56 EPC
Closest prior art

4. The board agrees to appellant's I position that the closest prior art is represented by documents (1) or (43), wherein the prospect of enzyme engineering is explained in general, including the role of X-ray crystallography, gene modification and computer modelling of protein structure and folding, without, however, making reference to subtilisins.

Problem to be solved and its solution

5. The technical problem to be solved on the basis of this teaching is to apply enzyme engineering to subtilisins, a technique which is possible only if one first identifies correct sites for mutation among the about 275 sites of the mature protein. The solution to this problem as embodied by the claims of the main request lies in the identification of possible sites for mutation among the about 275 sites of the mature protein, at which sites one can substitute and obtain variation in properties of the enzyme in ways that are very likely to be useful in terms of certain physical

features (eg, pH activity, oxidation stability), while still retaining enzyme function. In view of Examples 17 to 20 of the patent in suit, the further experimental evidence provided by appellant I before the examining division on 6 September 1986 (document (49)) and before the opposition division on 16 March 1994 (document (50)), the board is satisfied that the above problem has been solved by the identification of 11 such sites.

6. Appellants II and III argue in substance that a series of prior art documents (see section VIII supra) relating to the 3D structure of subtilisins indicated the positions recited in claim 1 as "promising candidates" because the claimed sites were within or near "binding pockets" and the skilled person knew that replacing an amino acid within or near the "binding pocket" of an enzyme would have resulted in altered properties of the enzyme. In the board's view, firstly it has to be emphasised that the problem the patent in suit purports to solve does not merely consist in obtaining "altered properties of the enzyme" but choosing residues which upon substitution would affect properties of the enzyme in useful ways, while substantially preserving the enzymatic function.

7. Furthermore, none of the 14 documents relating to the 3D structure of subtilisins and showing that certain residues are within or in proximity of binding pockets tells the skilled person that replacement at these sites would affect the properties of the enzyme in useful ways without substantially affecting enzymatic activity. Given that position Met+222 is the one which had been evaluated best in the prior art (document (5)), substitution at this position is discussed first,

in respect of inventive step. It lies in proximity of an active site (see document (5), page 5337, under the heading "Discussion") and this makes it sufficiently representative of residues having this property. Any conclusion reached by the board in connection with this position then applies a fortiori to the remaining positions recited in claim 1.

8. Document (5) teaches that a slight change at position Met+222 (-S- Y -SO-) substantially inactivates the enzymatic activity of subtilisin (see Fig. 1). Further, document (7), page 4301, suggests that the ã-sulphur atom at position +222 of subtilisin (and position +42 of chymotrypsin) is sacrosanct for enzyme activity. These facts do not confer on the board the impression that the prior art literature presents position Met+222 as a "promising candidate". Thus, while the board cannot accept appellant's I proposition that the skilled person expected a "catastrophic" loss of activity by replacement of conserved amino acids within or in proximity of "binding pockets", his/her expectation of obtaining useful variations in properties of the enzyme, while still retaining enzyme function, was poor at best. This is further supported by documents (2) and (3), which, according to appellants II and III, demonstrate that it was possible to select advantageous sites in tRNA synthetase and ß-lactamase, respectively: also in these cases a substantial loss of enzyme activity takes place upon altering residues near or within the binding pocket. Therefore, appellants' II and III proposition that a site looks interesting merely because it seems to be involved eg in substrate binding, is not supported by the prior art literature.

9. It was also argued by appellants II and III that a series of documents correlated chemical modifications of both subtilisins BPN' and Carlsberg at the specific sites recited in the claims with altered catalytic parameters. However, the board observes that none of these documents unambiguously suggests that oxidation, nitration, iodination, glutarylation or succinylation occurred stoichiometrically and/or uniquely at one site. Therefore, it could not be established with sufficient certainty whether or not residual activity of a chemically modified subtilisin was due to a portion of unreacted enzyme or to modified residues. For instance, during oxidation of Met+222 with H_2O_2, there is less than one equivalent oxidizing agent consumed (see document (5), Table I, according to which there is only 0.6 residues methionine sulfoxide in oxidized subtilisin). On page 267 of document (12), it is stated that the maximal change in rate of hydrolysis was obtained when a number of Tyr residues falling between one and two had been modified. Document (12) on page 270 states that the marked change in enzymatic behaviour was caused by modification of seryl and/or threonyl residues. Therefore, the conclusion cannot be drawn that all these documents unambiguously establish a correlation between a chemical modification at **one** particular site of subtilisin with altered catalytic parameters. Consequently, they do not render obvious the selection of Met+222 recited in claim 1. As said above, the prior art gets closest to the Met+222 site. When accepting inventive step for an alteration of this site, the same reasoning applies a fortiori for the other sites recited in claim 1.

10. The differences between the present situation and the

one dealt with in decision T 964/92 (OJ EPO 1997, 408) are that in the latter case, the actual technical contribution by the disclosure of that patent was found to be the successful completion of an experiment announced in an oral disclosure (ibidem, point 11). The patent in suit provides experimental evidence that some positions (Met+222, Gly+166 and Gly+169) of subtilisins "work" when replaced, while no such experimental evidence is to be found for the remaining positions (Glu+156, Ser+33, Phe+189, Tyr+217 and Ala+152) recited in claim 1 of the patent in suit. However, compared with the situation dealt with in decision T 964/92 (loc. cit.), the present case is characterized by one further measure the skilled person has **of necessity** to take in order to arrive at the claimed subject-matter (see point 5 supra), which measure contributes to the inventive step, namely to identify the correct sites for mutation among the about 275 sites of the mature protein. Therefore, the conclusion cannot be drawn that the actual technical contribution by the disclosure of the patent in suit is the successful completion of experiments foreshadowed at a theoretical level. Rather, the board has to evaluate whether or not the prior art comprised pointers to these 11 sites, and it has turned out that it did not.

11. It is worth remarking that the fact that finding "respective positions" was within the reach of the skilled person within the meaning of Article 83 EPC (see point 3 supra) does not render obvious the problem of "identifying correct sites for mutation among the about 275 sites of the mature protein" (see point 5 supra). This is because finding "respective positions"

does not automatically imply that these "respective positions" are good for the purpose of substituting and obtaining variation in properties of the enzyme in ways that are very likely to be useful in terms of certain physical features (eg, pH activity, oxidation stability), while still retaining enzyme function.

12. In view of the above findings, it must be concluded that the prior art comprised no pointers to the 11 sites recited in claim 1. Since the claims of the main request all directly or indirectly rely upon this inventive feature, the subject-matter of the claims pertaining to this request fulfils the requirements of Article 56 EPC.

Reimbursement of the appeal fee (Article 113, Rule 67 EPC)

13. Appellant I maintains that the opposition division violated the right to be heard (Article 113 EPC) by preventing him from arguing a broader case (see end of Paragraph VII supra) and hence requests the reimbursement of the appeal fee (Rule 67 EPC). The board, however, is unable to see such violation. In fact, the opposition division already expressed in its communication of 17 September 1992 (see point 7) its "most serious" concern that the broad wording of claim 1 might not satisfy the requirements of Article 56 EPC. Appellant I was given the opportunity to comment on this position of the opposition division (ibidem, point 8). Counterarguments were presented twice by appellant I (submissions of 1 February 1993 and 14 March 1994). Moreover, the minutes of the oral proceedings before the opposition division state under point 7: "After the parties concerned **had the**

opportunity to present their comments, the Chairman informed the parties that the Opposition Division considers the Main Request and the four Subsidiary Claim Requests unacceptable under Article 56" (emphasis added). All these facts do not confer on the board the impression of a breach of appellant's I right to be heard.

Order

For these reasons it is decided that:

1. The decision under appeal is set aside.

2. The case is remitted to the first instance with the order to maintain the patent on the basis of the following documents:

   (a) claims 1 to 20 submitted as main request during oral proceedings on 6 July 1999,

   (b) description pages 3 to 20 submitted during oral proceedings on 6 July 1999,

   (c) Figures 1 to 16 of the patent as granted.

3. The request for reimbursement of the appeal fee by appellant I is refused.

The Registrar: The Chairwoman:

U. Bultmann U. Kinkeldey