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**D E C I S I O N**  
of 26 November 1997

**Case Number:** T 0758/94 - 3.3.4  
**Application Number:** 87900930.6  
**Publication Number:** 0254735  
**IPC:** C12N 9/56

**Language of the proceedings:** EN

**Title of invention:**

Thermally stable and pH stable subtilisin analogs and method  
for production thereof

**Patentee:**

AMGEN INC.

**Opponent:**

- (01) Novo Industri A/S  
(02) Gist-Brocades N.V.  
(03) Unilever N.V.

**Headword:**

Subtilisin analogs/AMGEN

**Relevant legal provisions:**

EPC Art. 56

**Keyword:**

"Inventive step (yes) - after amendments"

**Decisions cited:**

T 0292/85

**Catchword:**



Case Number: T 0758/94 - 3.3.4

**D E C I S I O N**  
of the Technical Board of Appeal 3.3.4  
of 26 November 1997

**Appellant I:** (Opponent 01) Novo Industri A/S  
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**Decision under appeal:** Interlocutory decision of the Opposition Division  
of the European Patent Office posted 25 July 1994  
concerning maintenance of European patent  
No. 0 254 735 in amended form.

**Composition of the Board:**

**Chairwoman:** U. M. Kinkeldey  
**Members:** L. Galligani  
S. C. Perryman

## Summary of Facts and Submissions

I. The appellants (opponents 01 and 03) lodged an appeal against the interlocutory decision of the opposition division issued on 25 July 1994 whereby the European patent No. 0 254 735, which they had opposed under Article 100(a) and (b) EPC, was maintained on the basis of amended claims 1 to 26. The amended claims differed from the claims as granted in that in claims 1, 5, 10, 15, 25 and 26 the subtilisin analog referred to was qualified as "non-naturally occur[r]ing". The opposition division held that the said amendment, which was seen as formally acceptable under the terms of Articles 123(2)(3) and 84 EPC, restored novelty in respect to the following documents:

- (2) Jacobs et al., Nucl. Ac. Res., Vol. 13, 1985, pages 8913 to 8926;
- (6) Jany et al., Biol. Chem. Hoppe-Seyler, Vol. 366, 1985, pages 485 to 492;
- (7) Nedkov et al., Biol. Chem. Hoppe-Seyler, Vol. 366, 1985, pages 421 to 430;
- (8) Meloun et al., FEBS Letters, Vol. 183, 1985, pages 195 to 200.

The claimed subject-matter was also considered to be sufficiently disclosed and to involve an inventive step having regard to the cited state of the art, in particular document (8), which was seen as representative of the closest prior art, in combination with either one of the following citations:

- (1) Bornstein et al, Methods in Enzymology, Vol. XLVII, 1977, pages 132 to 145;

(16) Ahern et al., Science, Vol. 228, 1985, pages 1280 to 1284.

- II. On 7 August 1997, the board issued a communication wherein preliminary objections under Article 84 EPC were raised against the claims as maintained by the opposition division.
- III. With letter dated 26 September 1997, the respondents (patentees) filed three auxiliary claim requests.
- IV. Oral proceedings took place on 26 November 1997. During the oral proceedings a sole request was submitted, all previous requests being withdrawn. This request consisted of claims 1 to 3 together with an amended description. These claims correspond to claims 22 to 24 as granted and read as follows:

"1. A method for improving the thermal and pH stability of a Bacillus subtilisin having an amino acid sequence comprising an Asn-Gly sequence comprising the step of replacing a residue in said Asn-Gly sequence with a residue of a different amino acid.

2. A method as recited in claim 1 wherein said residue is the asparaginyl residue at the position in the amino acid sequence of the Bacillus subtilisin which corresponds to position 218 in the amino acid sequence as disclosed Table 1.

3. A method for improving the thermal and pH stability of a Bacillus subtilisin having an amino acid sequence comprising an Asn-Gly sequence comprising the step of deleting a residue in said Asn-Gly sequence."

V. The appellants essentially argued that, while it could be acknowledged that the technical effect of an improved thermal stability of subtilisin, in particular of the aprA subtilisin, was achieved by replacing the asparagine (Asn) residue in position 218 with a serine (Ser) residue, the same technical effect as well as the effect of improving pH stability was not achievable over the whole area claimed. The skilled person knew that an amino acid replacement was a critical matter and that no general conclusions could be drawn by just one specific successful replacement. As a matter of fact, the following post published art document:

(13) P.N. Bryan et al, Proteins: Structure, Function, and Genetics, Vol. 1, 1986, pages 326 to 334

demonstrated that other types of substitutions in position 218, eg with cysteine or alanine, provided the opposite result, ie a lower thermal stability. Moreover, the same document explained the effect of enhanced stability with improvements in the hydrogen bonding within the subtilisin molecule, not, as done by the patent in suit, with the cyclization at the level of the Asn-Gly sequence. Under these circumstances, inventive step could not be acknowledged for the broad claims on file. The present case was different from that of decision T 292/85 (OJ EPO 1989, 275) because in the latter the general teaching was sufficient to allow the skilled person to achieve the technical effect over the whole area which was claimed.

The appellants observed also that the generally claimed method lacked an inventive step over the combination of document (16) with document (1). In fact, the first taught the destabilising effect of the Asn residues in proteins and invited the skilled person to replace them with other amino acid residues. The second document drew the skilled person's attention to the fact that

cyclization occurred in proteins at the level of the Asn-Gly sequence. This readily invited the skilled person to replace in an Asn-Gly sequence Asn with a different amino acid.

- VI. In the respondents' view the closest prior art in the present case was represented by the following document:

(12) EP-A-0 130 756.

This document dealt specifically with muteins of subtilisin and proposed eg on page 19 (see page 8 of the B-document) a number of amino acid substitutions possibly resulting in improved oxidation stability and pH profiles. None of these was a modification at the level of an Asn-Gly sequence. The method claimed was the result of a surprising finding which also document (16) or document (1) did not render obvious.

- VII. The appellants requested that the decision under appeal be set aside and the patent be revoked.

The respondents requested that the decision under appeal be set aside and that the patent be maintained on the basis of the sole request submitted at oral proceedings on 26 November 1997.

## Reasons for the Decision

### *Formal requirements (Articles 123 and 84 EPC)*

1. No formal objections under Articles 123 and 84 EPC were raised by the appellants and none is seen by the board.

*Inventive step (Article 56 EPC)*

2. As neither sufficiency of disclosure nor novelty of the claimed subject-matter were contested by the appellants, the only relevant issue to be discussed is inventive step. For this purpose, the board, in agreement with the respondents, sees in document (12) the closest prior art as it deals with modifications of the primary structure of subtilisins in order to achieve an improvement in its stability and pH profiles. To this extent the document proposes a number of amino acid replacements in different positions, none of them involving an amino acid in any of the available Asn-Gly sequences.
3. In the light of document (12), the problem to be solved was the finding of amino acid replacements which result in an improved thermal and pH stability of a *Bacillus subtilisin*.
4. As a solution thereto the claims on file propose a method comprising the step of replacing a residue in an Asn-Gly sequence of a *Bacillus subtilisin* with a residue of a different amino acid (claim 1) or the step of deleting a residue in the said Asn-Gly sequence (claim 3). The patent specification provides the specific example of the replacement of the Asn in position 218 with a Ser in the aprA subtilisin. This is shown to result in a greater stability of subtilisin than the corresponding wild type enzyme. The patent in suit also indicates further positions in subtilisins in which an amino acid residue substitution is expected to improve stability (cf page 22 of the specification), no experimental results being provided. During the course of the opposition proceedings, the respondents had provided a declaration of Dr Linda O. Nahri dated 22 February 1989 showing the increased thermal stability of the aprA subtilisin in which the Asn

residue in position 109 or in 218 or in both positions was replaced by Ser.

5. The appellants object, with reference to document (13), that the claimed technical effect is not achievable over the whole area claimed. The board observes that the said document, while confirming the effectiveness of the Asn replacement in position 218 with Ser, does not cast doubts on the validity of the general proposition made by the patent in suit. This is because, even if the same document shows that the replacement of the same Asn residue with Cys or Ala results in a lower relative stability based on thermal inactivation, it does not assign a unique role for position 218 and/or for Ser replacement in the selection of thermostable subtilisins. Thus, although part of the conceivable embodiments falling within the claims may turn out to be less effective than others or even to be counterproductive, the evidence on file makes it plausible that, notwithstanding the unpredictability of the effect of an amino acid substitution or deletion in a protein, a number of possible variations of the subtilisin primary structure according to the claimed method would indeed result in an improved stability of the molecule. Therefore, the board is satisfied that the underlying technical problem is solved by the claimed solution in its broad outline.
  
6. The skilled person, faced with the stated technical problem, would not have derived from document (12) any hint or suggestion in respect to the replacement or deletion of an amino acid residue in an Asn-Gly sequence of subtilisin. The relevant question is thus whether the skilled person would have readily derived such an hint or suggestion in this respect from any other of the cited documents. The appellants made reference in particular to documents (16) and (1).



7. Document (16) examines the mechanisms of irreversible thermal inactivation of enzymes, in particular of lysozyme. The document observes inter alia that Asn residues in enzymatic proteins, as they undergo deamination, contribute to the irreversible thermoinactivation. The document thus suggests that the replacement of Asn with another amino acid residue should enhance thermostability. The document, however, does not point to any Asn residue in any particular sequence within the enzyme molecule as being the source of thermal or pH instability.
8. Document (1) discusses the cleavage at Asn-Gly bonds with hydroxylamine in proteins, in particular in collagen. In this context, the document draws the reader's attention to the fact that cyclization of the asparaginyl-glycyl bond can take place and that there are factors such as low pH which can foster it. The document, however, does not refer to any problem of thermal or pH stability of a protein in relation to this cyclization reaction.
9. In the board's judgement, the skilled person would not have derived from document (16) or from document (1), alone or in combination with each other or with document (12), the idea of replacing or deleting an amino acid residue specifically in an Asn-Gly sequence of subtilisin. This is because, on the one hand, there are some seventeen Asn residues in the primary sequence of subtilisin and thus, without foreknowledge of the claimed invention, it would not have been possible, on the basis of document (16), to readily focus the attention on those in the Asn-Gly sequence. The number of Asn residues also would have thrown doubt on replacement of these really being a way to increase stability while maintaining protease properties. On the other hand, the study in document (1) is directed to the susceptibility of the Asn-Gly bonds in proteins to

cleavage with hydroxylamine, nothing being said about thermostability or pH stability. Thus, the skilled person would not even have taken this document into account when faced with the stated technical problem.

10. For these reasons, in the board's judgement, the subject-matter of claims 1 to 3 involved an inventive step and, consequently, the respondents' sole request is allowable.

*The adaptation of the description to the claims*

11. At oral proceedings, the respondents provided an amended description (hand-numbered pages 1-24) adapted to claims 1 to 3. The amended description now indicates on hand-numbered pages 1 to 5, 18 and 23 that the claimed invention relates to methods for improving the thermal stability and pH stability of **Bacillus subtilisins**, the remaining pages being unchanged. Although no formal objections under Article 123(2) EPC were raised by the appellants, these wished further clarifications on hand-numbered pages 6 and 14, these being, respectively, a reference to the method on line 2 of page 6 and the replacement of the words "the stabilisation" with the expression "a method for improving the thermal and pH stability" in Example 7. The board considers that no further amendments are necessary as it is clear now that the patent specification is directed to a method for improving the thermal and pH stability of subtilisin and that nothing else is meant.

The figures as referred to in the description of course remain unchanged.

Order

For these reasons it is decided that:

1. The decision under appeal is set aside.
2. The matter is remitted to the first instance with the order to maintain the patent on the basis of claims 1 to 3 (hand-numbered page 25) and the amended description (hand-numbered pages 1-24) forming the sole request submitted at the oral proceedings on 26 November 1997.

The Registrar:

  
D. Spigarelli

The Chairwoman:

  
U. M. Kinkeldey

