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D E C I S I O N
of 5 October 1998

Case Number: T 0916/94 - 3.3.4

Application Number: 83307840.5

Publication Number: 0114506

IPC: C12N 15/00

Language of the proceedings: EN

Title of invention:
Methods of purification and reactivation of precipitated heterologous proteins

Patentee:
Genentech, Inc.

Opponents:
Boehringer Mannheim GmbH Patentabteilung
Behringwerke Aktiengesellschaft
Eli Lilly and Company
Chiron Corporation

Headword:
Reactivation/GENENTECH

Relevant legal provisions:
EPC Art. 54, 56, 84, 123

Keyword:
"Main request and first to third auxiliary requests - novelty (yes)"
"Inventive step (no)"

Decisions cited:
T 0597/92, T 0435/91, T 0923/92, T 0626/91

Catchword:
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Beschwerdekammern | Boards of Appeal | Chambres de recours

Case Number: T 0916/94 - 3.3.4

D E C I S I O N
of the Technical Board of Appeal 3.3.4
of 5 October 1998

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Decision under appeal: **Decision of the Opposition Division of the European Patent Office posted 26 September 1994 revoking European patent No. 0 114 506 pursuant to Article 102(1) EPC.**

Composition of the Board:

Chairman: L. Galligani
Members: F. L. Davison-Brunel
C. Holtz
R. E. Gramaglia
S. C. Perryman

Summary of Facts and Submissions

I. The appeal lies from the decision of the opposition division issued on 26 September 1994 whereby the European patent No. 0 114 506, which had been opposed under the terms of Article 100(a) and (b) EPC by four parties (opponents 1 to 4), was revoked pursuant to Article 102 EPC. The priority date of the patent was 22 December 1982.

II. The decision related to eight claim requests, each in two versions, one for all contracting States except Italy (non-IT States) and one for Italy (IT). A total of sixty three citations was taken into account by the opposition division which had also taken evidence by hearing two witnesses, namely Prof. A. Goldberg and Dr W. F. Prouty. Of these citations, the following are referred to in the present decision:

(3) Mozhaev V. V. et al., Enzyme Microb. Technol., September 1982, vol. 4, pages 299 to 309;

(8) Shine J. et al., Nature, June 1980, vol. 285, pages 456 to 461;

(11) Williams D. C. et al., Science, 5 February 1982, vol. 215, pages 687 to 689;

(12) Wetzel R. et al., in Cellular Response to Molecular Modulators, L. W. Mozes et al. eds., 1981, Academic Press, New York, N.Y., USA pages 251 to 270;

(14) Itakura K. et al., Science, 9 December 1977, vol. 198, pages 1056 to 1063;

(17) WO-A-83/04418, with earlier priority date of 7 June 1982, designating for a European patent the same European states as the patent in suit except Italy;

(18) Kleid D. G. et al., Science, 4 December 1981, vol. 214, pages 1125 to 1129;

(20) Anfinsen C. B., Science, 25 July 1973, vol. 181, pages 223 to 230;

(28) Presentation by Dr Norm Lin at the Parenteral Drug Association Genetic Engineering Mini-symposium at Chicago, Illinois (USA) on 1 April 1982.

(35) Prouty W. F. et al., J. Biol. Chem., February 1975, vol. 250, pages 1112 to 1122;

(44) Mitraki A. et al., Biotechnology, July 1989, vol. 7, pages 690 to 697.

III. The opposition division decided that none of the claim requests then on file satisfied the requirements of the EPC as they lacked either novelty, in particular having regard to documents (8), (14), (17), (18), or inventive step having regard to various combinations of documents.

IV. With the statement of grounds of appeal, the appellants (patentees) filed five additional citations together with a main claim request and three auxiliary claim requests, each in two versions for IT and for non-IT States, respectively. A declaration by Dr Norman Lin

was filed separately on 23 August 1995. Of the five additional citations, the following is referred to in the present decision:

(64) Marston F. A. O., Biochem. J., 1986, vol. 240, pages 1 to 12.

V. All respondents (opponents 01 to 04) filed their comments to the statement of grounds of appeal.

VI. On 8 April 1998 the board sent a communication to the parties under Article 11(2) of the rules of procedure of the boards of appeal with an outline of the points to be discussed and some preliminary remarks on possible formal objections.

VII. The appellants and respondents II and IV made further submissions in reply to the board's communication. Respondents IV filed a declaration by Dr Leo Lin. The appellants revised their claims requests in order to meet the formal objections raised by the board.

VIII. Oral proceedings took place on 5 October 1998. During oral proceedings a new main request and three auxiliary claim requests in two versions for non-IT States and IT were submitted in substitution of the requests on file.

Independent claims 1, 14 and 24 of the **main request** for non-IT States read as follows (in bold-type characters the amendments and in square brackets the deletions in comparison with the corresponding independent claims 1, 14 and 25 as granted):

"1. A method of obtaining biologically active protein from a refractile protein produced as a heterologous expression product in a host cell culture; wherein the method comprises separating insoluble refractile protein from non-refractile material, contacting the

refractile material with a strongly denaturing solution sufficient to solubilize it, and treating the protein while maintaining it in solubilized form so as to allow the heterologous protein to assume a biologically active conformation, said treatment comprising converting the strongly denaturing solution to a weakly denaturing solution, **and from thence converting the protein into a non-denaturing solution,** while maintaining said protein in solution, said protein treatment further comprising cleaving disulfide bonds of the solubilized protein followed by reformation of the disulfide bonds under conditions which allow the protein to assume a conformation capable of exhibiting biological activity."

"14. A method of obtaining biologically active protein **other than Met-prochymosin** from a refractile protein produced as a heterologous expression product in a host cell culture; wherein the method comprises separating insoluble refractile protein from non-refractile material, contacting the refractile material with a strongly denaturing solution sufficient to solubilize it, and treating the protein while maintaining it in solubilized form so as to allow the heterologous protein to assume a biologically active conformation, said treatment comprising replacing the strongly denaturing solution with a [**different**] weakly denaturing one, **having a chaotropic agent different from that used in said strongly denaturing solution, and from thence converting the protein into a non-denaturing solution,** while maintaining said protein in solution."

"24. A method of obtaining biologically active protein from a refractile protein produced as a heterologous expression product in a host cell culture; wherein the method comprises separating insoluble refractile protein from non-refractile material, **in such a way**

that the cellular debris fails to appear in conjunction with the refractile protein, contacting the refractile material with a strongly denaturing solution sufficient to solubilize it, and treating the protein while maintaining it in solubilized form so as to allow the heterologous protein to assume a biologically active conformation, said treatment comprising converting the strongly denaturing solution to a weakly denaturing solution, **and from thence converting the protein into a non-denaturing solution,** while maintaining said protein in solution, and effecting one or more purification procedures while the protein is in denaturing solution."

In the same claims of the **first auxiliary request** (non-IT States), the words "refold and" were inserted between "heterologous protein to" and "assume". Moreover, in claim 1 the expression "to assume a conformation capable of exhibiting biological activity" was changed to read "to assume a refolded conformation capable of exhibiting biological activity".

In the same claims of the **second auxiliary request** (non-IT States), it was specified that the "heterologous expression product" was "other than as fusion with a bacterial polypeptide".

In the same claims of the **third auxiliary request** (non-IT States), the amendments introduced in the first and second auxiliary requests were combined.

The main difference between the sets of claims for IT and those for the non-IT States was in respect of claim 14 which in the version for IT did not specify that the biologically active protein was "other than Met-prochymosin", and that the weakly denaturing one had "a chaotropic agent different from that used in said strongly denaturing solution".

IX. The appellants submitted in essence that the claimed method was novel over the disclosures of documents (8), (14), (17) and (18) as none of these went through a procedure as outlined in the claims.

As for inventive step, they argued essentially that at the priority date the technical situation relating to refractile bodies was complex and full of uncertainties. Reference was made in this respect to the later review articles (44) and (64). In 1982 the refractile bodies were seen either as a reservoir of abnormal proteins ready to be degraded (cf document (35)) or as a consequence of the production of insoluble fusion proteins. The skilled person did not expect that non-fused, normal proteins could aggregate into refractile bodies. As regards the insoluble fusion proteins, the general belief was that they had to be cleaved before they could be solubilised (cf eg document (8)). The skilled person regarded the refractile bodies as being nothing more than "an interesting curiosity" and did not expect that biologically active proteins produced in fused or unfused form could be recovered therefrom. It was the inventive merit of the patent in suit to show that this was indeed possible and to disclose a suitable unfolding-refolding process therefor. Two factors thus contributed to inventive step: (i) the realisation that normal active proteins (fused or unfused) could be obtained from the refractile bodies and (ii) the technology of denaturation-renaturation.

X. The respondents, apart from some formal objections (cf point 3 of the reasons infra), argued essentially that there were no meaningful technical differences between the operational steps disclosed in documents (8), (14) and (18) for recovering an active protein which had been expressed in a host cell culture in an insoluble form, this being inevitably refractile, and

the steps outlined in the claims at issue. Therefore, there was no technical contribution to the art by the patent in suit as the combinations of features recited in the claims were trivial combinations which could not justify novelty, much less so inventive step. In the view of respondents I, the disclaimer in claim 14 was not sufficient to establish novelty over document (17) as the disclosure of the latter document was not limited to the denaturation-renaturation of Met-prochymosin.

XI. The appellants requested that the decision under appeal be set aside and that the patent be maintained, for all designated States except Italy, on the basis of the main request or first to third auxiliary requests, as submitted in the oral proceedings, all amended by the disclaimer inserted into claim 14, as submitted in the oral proceedings, and, for Italy, the main request or first to third auxiliary requests as submitted in the oral proceedings.

The respondents requested that the appeal be dismissed.

Reasons for the Decision

The main request (claims for non-IT states and for IT)

Article 123(2)(3) EPC

1. The various amendments introduced in the claims at issue in comparison with the claims as granted are of a restrictive nature. This is in compliance with the requirements of Article 123(3) EPC.

2. In the board's judgement, none of the amendments results in the creation of subject-matter which extends beyond the content of the application as filed because:

   (a) The feature "and from thence converting the protein into a non-denaturing solution," (cf claims 1, 14 and 24), which was found in claim 22 as granted, finds its basis in the teaching in the application as filed of progressively reducing the strength of the denaturing solution so as to allow refolding of the protein in a weakly denaturing or non-denaturing milieu (cf eg claims 1, 4, 5, 27 as originally filed);

   (b) The feature "other than Met-prochymosin" (claim 14) is a disclaimer necessary in order to avoid anticipation by document (17), which in Example 1 describes the same method applied to Met-prochymosin;

   (c) The feature "having a chaotropic agent different from that used in said strongly denaturing solution," (cf claim 14) finds its basis on page 5, lines 6 to 8 and in the passage bridging pages 7 and 8 of the application as filed;

(d) The feature "in such a way that the cellular debris fails to appear in conjunction with the refractile protein," (cf claim 24) finds its basis on page 20, lines 6 to 9 of the application as filed.

Thus, no objections under Article 123(2) EPC arise.

Clarity (Article 84 EPC)

3. The respondents argued that many expressions used in the claims were not clear because, also in the light of the description, they did not have an unambiguous meaning. For example:

- it was not possible to clearly distinguish between "strongly denaturing" and "weakly denaturing" and between "weakly denaturing" and "non-denaturing";

- the distinction between "cellular debris" and "cellular fragments" was not clear;

- the exact meaning of terms "denaturation", "refractile", "different chaotropic agent" was not clear having regard to the broad definitions given in the patent specification.

4. The appellants observed that the terms and expressions objected to were those of the claims as granted and submitted that it was not possible to raise objections under Article 84 EPC against them in opposition-appeal proceedings. In any case, the definitions were given in the description of the patent in suit and these had to be looked at from the point of view of common sense.

5. The board observes that indeed most of the terms and definitions objected to are found in the claims as granted and thus their clarity cannot be put into discussion. However, although clarity under Article 84 EPC is not open to objection under the terms of Article 100 EPC, questions of clarity may affect the decision on issues under Article 100 EPC such as novelty or inventive step (cf eg decisions T 435/91, OJ EPO 1995, 188, T 923/92, OJ EPO 1996, 564 and T 626/91 of 5 April 1995). This may, for example, be the case when a broad or vague meaning attributed to a term in the description (the latter being used to interpret the claims according to Article 69(1)) does not allow a clear-cut distinction over the prior art.

6. In the present case, the following definitions provided in the patent in suit are noted:

(i) "**Refractile protein**" refers to a protein, "which **at some stage** of expression or purification, is visible by phase contrast microscope as a precipitate, regardless of the physical state of the protein at the time is referenced" (cf page 6, lines 12 to 15) (emphasis added).

(ii) "**Biologically active conformation**" refers to a conformation of the protein such as to ensure its activity in vivo or in vitro in a biological assay designed to test its functionality, its ability to elicit an immune response or to react with antibodies to the native protein (cf page 6, lines 27 to 28 and lines 33 to 37). This does not necessarily imply a refolding of the protein to a state identical to that of the native protein, as also a different conformation could result in a positive response in a functionality test.

(iii) "**Denaturing solution**" refers to a solution which contains a compound or material which, in aqueous solution and in suitable concentrations, is capable of changing the spatial configuration or conformation of proteins through alterations at the surface thereof, either through altering, for example, the state of hydration, the solvent environment, or the solvent-surface interaction. Examples are urea, guanidine hydrochloride (GuHCl), sodium thiocyanate (ST) and detergents such as SDS and Triton (cf page 6, lines 40 to 46). A "**strongly denaturing**" solution refers to a solution which will unfold a protein (eg GuHCl, urea and ST 4-9 M or detergents 0.01-2%) (cf page 6, lines 5963). "**Weakly denaturing**" solutions are those solutions which permit at least folding of a protein into the spatial conformation in which it finds itself when operating in its active form under endogenous or homologous physiological conditions, and also solubilizing any intermediate forms between the "denatured" form as would be found in a strongly denaturing solution, and the properly folded conformation (eg GuHCl, urea and ST 0.5-2 M (cf page 6, line 64 to page 7, line 11). This could also include low buffer concentrations (0.1 M or lower).

These definitions have to be taken into account when interpreting a prior art teaching in the substantive examination of the claimed subject-matter.

7. The expression "cellular debris", which was not contained in the claims as granted, is commonly used in the biological field (cf eg document (14), page 1062, left-hand column, line 5 from the bottom or document (18), page 1126, center column, line 10) and its meaning is thus clear to the skilled person. In the

context of claim 24, the purpose of the expression is to indicate that the operation of separation of the refractile protein from non-refractile material should be carried out in such a way that cellular material resulting from cell disruption is not found in the pellet under low speed centrifugation. In the board's view, this implies sufficiently clear instructions for a skilled person. Thus, no objection under Article 84 EPC arises.

8. As for the feature "having a chaotropic agent different from that used in said strongly denaturing solution," (cf claim 14), it clearly instructs the skilled person to replace the strongly denaturing chaotropic agent with a different agent which has weakly denaturing properties (cf page 4, lines 14 to 15 and page 5, lines 6 to 8 of the patent specification). The latter, according to the definition given in the description (cf point 6, (iii) supra), could simply be a low concentration buffer (0.1 M or lower). No objection under Article 84 EPC is seen by the board.

Novelty (Article 54 EPC)

9. As regards the novelty issue, at oral proceedings reference was made by the respondents to documents (8), (14), (18) as well as to document (17) (this latter under Article 54(3)(4) EPC). They argued that the said documents disclosed a method of obtaining a biologically active protein from a refractile protein produced as a heterologous expression product in a host cell culture which was undistinguishable from the method claimed in the patent in suit.

10. As regards document (17), Example 1 therein, which is found also in its first priority document dated 7 June 1982, discloses indeed a method of purification of Met-prochymosin from an insoluble aggregate which comprises

the same steps as the method of claim 14 at issue, ie solubilisation of the protein with a denaturing agent such as urea or GuHCl, and renaturation to a biologically active form by reducing the concentration of the said agent by dialysis against a low concentration buffer. The teaching of the said example is strictly limited to Met-prochymosin. Nothing in the said document indicates to the skilled reader that the same purification method can analogously be applied to other precursor forms of chymosin, much less so to any other protein. As the disclosure of document (17) is excepted by mean of a disclaimer in the set of claims for all non-IT states, the novelty of their subject-matter is affirmed. As IT is not a designated state in document (17), there is no need for such a disclaimer in the separate set of claims for IT.

11. As regards the references (8), (14), (18), the board - for the reasons given hereinafter - is of the view that they did not unambiguously disclose for a skilled person a method as claimed. A method claim is directed to an activity (here: the recovery of a biologically active protein from a refractile body) which might comprise - as in the present case - the execution of a series of operational steps, each of them having an effect on the next step to be executed. One should avoid reading with hindsight into a document technical information or effects which is (are) not unambiguously revealed therein.

12. Document (8) describes the expression in E.coli of ß-endorphin in fused form. The hybrid protein is reported to be insoluble and to be recoverable from a high speed pellet of cell extracts. To this extent, the pellet is dissolved in 6 M GuHCl, the extracted fusion protein is chemically modified, dialysed and cut to free ß-endorphin which is tested for biological or immunological activity, and further purified. The said

document does not indicate that the insoluble protein occurred in refractile bodies. An insoluble protein cannot be equated to a refractile protein as the latter is a particular form of aggregation of insoluble proteins which is visible at some stage by phase contrast microscopy (cf point 6, item (i) supra). Moreover, the document, although describing that the desired protein is extracted from the pellet with a denaturing agent and subsequently dialysed, does not convey a structured technical teaching of a denaturation-renaturation process directly centred on the conformational changes of the protein.

13. Also in document (14) the expressed fusion protein (somatostatin) is insoluble and is found in the pellet from the first low speed centrifugation. Nothing is said about the occurrence thereof in refractile bodies. The protein is solubilized in 6 M GuHCl or 8 M urea or 2% SDS or 70% formic acid and at the same time treated with CNBr to free somatostatin, diluted tenfold in water, assayed radioimmunologically and further purified. As in the case of document (8), although some experimental steps correspond to operational steps of the method of the claims at issue, it cannot be said that document (14) discloses a denaturation-renaturation method with emphasis on the conformational changes of the protein to be extracted.

14. Document (18) reports that the expressed fusion protein (part of the VP3 protein of FMDV) is visualized as a refractile body and recovered in the cellular debris of lysed cells. The said protein is purified by two successive SDS PAGE runs in 8 M urea, the protein containing bands being recovered by electroelution or pulverization. The slurry is taken up in a buffer with 0.1% SDS and ß-mercaptoethanol and heated at 100°C for 5 minutes in order to enhance solubilization of the protein. The product is immunologically tested and

found active. In the board's view, a SDS-PAGE run in urea cannot technically be equated to a treatment of a refractile protein, while in solution, with a strong denaturant, in spite of the fact that the protein during the run is in solubilized form. Thus, also in this case, although the subject protein is first treated with a denaturing agent and then recovered in a biologically active form with a buffer, it cannot be said that the document conveys a structured technical teaching of a denaturation-renaturation process directly centred on the conformational changes of the protein.

15. For these reasons, the documents (8), (14) and (18) are not considered to affect the novelty of the claims at issue. It has not been argued that the novelty of the claims is affected by any of the other documents on file.

Inventive step (Article 56 EPC)

16. In the board's view, the most appropriate starting point for an inventive step analysis is the prior art knowledge that polypeptides produced as heterologous expression products in a host cell culture in some instances were sequestered into refractile bodies. At least three prior art documents on file explicitly reported this, namely document (18), already treated above (cf point 14 supra) as well as documents (11) and (28). The latter two describe the presence of refractile bodies in E.coli cultures producing the insulin chain chimeric proteins (document (11), see in particular Figure 2)) and thymosin and growth hormone (document (28), see in particular slides 6, 6a and 7).

17. In the light of the said prior art knowledge, the problem to be solved was finding a method for recovering a biologically active protein from the said refractile bodies.

18. As a solution thereto, the methods according to claims 1, 14 and 24 are proposed. These are three variations of a method essentially based on the extraction of the recombinantly expressed protein from the isolated refractile bodies by means of a strongly denaturing solution and subsequent renaturation (also partial; cf definition in point 6, item (ii)) to a biologically active conformation by changing the solution into a weakly-denaturing or non-denaturing solution (cf in particular claim 14). As shown in the patent specification, this method results in the recovery of biologically active proteins.

19. The key questions are what solution(s) to this problem, if any, the skilled person would have derived from the prior art using only his or her ordinary skill, and whether such solution(s) fall(s) under what is claimed.

20. The appellants submitted that the inventive contribution to the art by the patent in suit was the realization that refractile bodies were not merely a reservoir of abnormal proteins and thus useless, but that biologically active proteins could be recovered from them by way of a controlled process of denaturation-renaturation. In their view, the claims provided a solution to a fundamental problem in recombinant DNA technology and had to be worded broadly in order to outline the basic operational steps valid for all proteins. Only with hindsight could the proposed solution be regarded as being simple and easy. The technical situation in relation to the refractile bodies was not at all simple (cf later review articles (44) and (64)). In their view, the solution proposed

had brought certainty in an area of great uncertainty as nothing in the prior art suggested to the skilled person that protein sequestered into refractile bodies could be renatured.

21. However, the skilled person knew at the priority date that recombinantly produced proteins, in particular fusion proteins, were frequently found to occur in insoluble form in the host cell (see eg documents (8), (12), (14)). The observation had been made that in some instances this was in the form of refractile bodies (see documents (11), (18) and (28)). The latter were not considered merely "an interesting curiosity" which did not invite the skilled person to take action. Rather attempts were made to recover therefrom the desired product (cf document (18)). The commonly used approach for recovering the desired protein from the insoluble cellular material was to treat the pellet or the refractile bodies under denaturing conditions (eg with GuHCl or urea) so as to solubilize the protein and then proceed to its further purification and assay (cf documents (8), (14), (18)).

22. In view of this, the board cannot agree with the appellants' view that the skilled person, faced with refractile bodies, would have shown no special interest in them or even discarded them. On the contrary, as the prior art shows, the skilled person would have readily attempted to recover a biologically active protein therefrom. For this, the skilled person had basically only one way open, namely that indicated in the prior art (cf eg document (18)), ie treating the refractile bodies isolated by centrifugation with a solubilising agent, further purifying and assaying the solubilised protein. The skilled person was aware of the fact that during these operations the protein had to be treated with care in order to ensure the recovery of a biologically active form of the desired protein. To

"treat with care" involved the skilled person avoiding - especially in the case of labile proteins - drastic conformational changes which could lead to loss of biological activity. This was a known basic principle in protein chemistry and purification (cf document (20)). The principles governing the folding of protein chains, the denaturation-renaturation processes and the cleavage/reformation of disulfide bonds were of course well known to the skilled person (cf eg documents (3) and (20)).

23. Therefore, the skilled person, having once conducted the operation of solubilisation of the desired protein under denaturing conditions, would have readily proceeded to its further purification by diluting or eliminating the solubilising (denaturing) agent as there would have been no reasons to continue to operate in an environment encouraging conformational changes. As for the following purification step, the skilled person would have had a series of known methods to choose from. In any case, whatever the method chosen, common sense would have dictated to him or her to operate in an environment permitting reversible change back to an active conformation of the protein, with a view to assaying its biological activity.

24. In the board's judgement, the claims at issue, in particular claim 14, merely outline in a structured manner a series of general operational steps which, for the reasons given above, would have been taken by the skilled person. The emphasis put in the claims in functional language on the need to allow the heterologous protein to assume a biologically active conformation cannot per se contribute to inventive step because this was plain for a person of ordinary skill.

25. For these reasons, an inventive step is to be ruled out. Consequently, the main request is not allowable under Article 56 EPC.

First auxiliary request (claims for non-IT states and for IT)

26. In this request, the independent claims emphasize that the solubilised heterologous protein should be allowed to refold and assume a biologically active conformation. As already stated above (cf point 6, item (ii)), this does not imply a return to a conformational state identical to that of the native protein. The reasons given above for denying inventive step of the main request fully apply to this request which is not allowed for the same reasons.

Second auxiliary request (claims for non-IT states and for IT)

27. In this request, the independent claims merely exclude the application of the method to the recovery of proteins fused with bacterial polypeptides. The claims still cover other kinds of fusion proteins. Apart from the fact that here the attempt is made to substantiate inventive step by way of a disclaimer, which according to the case law (see eg T 597/92, OJ 1996, 135) cannot be done, the reasons for denying inventive step of the main request apply also here. Thus, also this request is not allowable under Article 56 EPC.

Third auxiliary request (claims for non-IT states and for IT)

28. In this request, the amendments introduced in the first and second auxiliary requests were combined. The reasons given above apply equally here and thus this request is not allowed under Article 56 EPC.

Order

For these reasons it is decided that:

The appeal is dismissed.

The Registrar: The Chairman:

D. Spigarelli L. Galligani