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Case Number: T 0063/17 - 3.3.04

Application Number: 09787605.6

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Language of the proceedings: EN

Title of invention:
Stable immunogenic protein having multiple cysteine molecules, process therefore and composition thereof

Applicant:
Bharat Biotech International Limited

Headword:
Stable Plasmodium vivax protein/BHARAT

Relevant legal provisions:
EPC Art. 56, 106, 107, 108
EPC R. 99
RPBA Art. 12(4)

Keyword:
Admissibility of appeal - (yes)
Amendments - allowable (yes)
Inventive step - (yes) non-obvious alternative

Decisions cited:

Catchword:

Beschwerdekammern
Boards of Appeal
Chambres de recours

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Case Number: T 0063/17 - 3.3.04

D E C I S I O N
of Technical Board of Appeal 3.3.04
of 4 March 2020

Appellant: Bharat Biotech International Limited
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Decision under appeal: **Decision of the Examining Division of the European Patent Office posted on 8 August 2016 refusing European patent application No. 09787605.6 pursuant to Article 97(2) EPC.**

Composition of the Board:

Chairwoman G. Alt
Members: O. Lechner
R. Romandini

Summary of Facts and Submissions

I. The appeal filed by the applicant (appellant) lies from the examining division's decision refusing European patent application No. 09 787 605.6. The application was filed as an international application under the PCT and entered the European phase on 12 December 2011 (hereinafter the "application as filed" or "application"). The title of the application is "*Stable immunogenic protein having multiple cysteines molecules, process therefor and composition thereof*".

II. In the decision under appeal, the examining division held that claim 1 of the main request, as filed during oral proceedings, did not comply with the requirements of Article 84 EPC because essential technical features, such as the "*definition of the codon optimized sequence of the protein(s), the regulatory and promoter components of the expression construct and the suitable strain of E. coli*", were missing (see point 14).

The subject-matter of claims 1 to 3 was held to lack inventive step (Article 56 EPC) because the skilled person, starting from document D1 as the closest prior art, would have arrived at it by routine experimentation in the light of documents D2, D3 and D4 (see point 13 of the decision under appeal).

III. With the statement of grounds of appeal, the appellant re-filed the main request dealt with in the decision under appeal and submitted four documents.

IV. The board issued three communications pursuant to Article 15(1) RPBA, in which it made observations *inter alia* concerning clarity and inventive step.

V. The appellant replied to the communications and filed two amended claim requests.

VI. Oral proceedings were held on 4 March 2020, during which the appellant filed a new main request and withdrew all other claim requests.

At the end of the oral proceedings the chair announced the board's decision.

VII. The sole independent claim of the main request reads as follows:

"1. Process for expression, purification and refolding of recombinant Plasmodium vivax Duffy binding protein region II (rPvRII) having multiple cysteines useful for the prophylaxis of malarial infection in mammals, the process comprising the following steps:
a) culturing the host E. coli cells containing a desired recombinant gene construct comprising a codon optimized gene sequence of rPvRII,
wherein rPvRII has the structure:

MDHKKTISSAIINHAFLQNTVMKNCNYKRKRRERDWDCNTKKDVCIPDRRY
QLCMKELTNLVNNTDTNFHRDITFRKLYLKRKLIYDAAVEGDLLLKLNNYR
YNKDFCKDIRWSLGDFGDIIMGTDMEGIGYSKWENNLRSIFGTDEKAQQRR
KQWWNESKAQIWTAMMYSVKKRLKGNFIWICKLNVAVNFFIPQIYRWIREW
GRDYVSELPTEVQKLKEKCDGKINYTDKKVCKVPPCQNACKSYDQWITRKK
NQWDVLSNKFISVKNAEKVQTAGIVTPYDILKQELDEFNEVAFENEINKRD
GAYIELCVCSVEEAKKNTQEWTHHHHHH

to produce cells in high density
b) inducing expression of rPvRII in E. coli as inclusion bodies,

c) harvesting the cells and isolating the said inclusion bodies,
d) separating rPvRII from inclusion bodies by repeated sequential washing with washing with a buffer, the buffer used being a low salt buffer consisting of 5-50 mM Tris, 2-25 mM EDTA, 50-100 mM sodium chloride, 1-6 M urea, 0.1-1% Triton X-100, pH 6.5-7.5; followed by washing with a high salt buffer containing 5-50 mM Tris, pH 6.5-7.5 and 0.5-1 M sodium chloride;
and solubilizing with chaotropic agents comprising guanidine hydrochloride at 6-8 M concentration,
e) purifying the protein by subjecting to metal-chelate affinity chromatography,
f) re-folding of the purified rPvRII obtained in step e) by employing a buffer containing a detergent - dextrin mixture, a co-solvent and a redox pair, wherein the detergent used is Triton X-100 at a concentration of 0.1-1.0 mM, a dextrin at a concentration of 5-20 mM, L-Arginine as a co-solvent being added in a concentration of 0.4-0.8 M and a redox pair comprising L-cysteine - cystamine dihydrochloride; followed by
g) further purifying the desired protein by removing impurities by subjecting to chromatography."

VIII. The following documents were referred to in the appeal proceedings:

D1: Syed Shams Yazdani, et al., "*A high cell density fermentation strategy to produce recombinant malarial antigen in E. coli*", Biotechnology (2004), Vol. 26, No. 24, pages 1891-1895

D2: Palmer I., et al., "*Preparation and Extraction of Insoluble (Inclusion Body) Proteins from Escherichia coli, Current Protocols in Protein Science (2004)*", Chapter 6, pages 6.3.1-6.3.18

D3: Wilharm E., et al., "*Generation of catalytically active granzyme K from Escherichia coli inclusion bodies and identification of efficient granzyme K inhibitors in human plasma*", Journal of Biological Chemistry (1999), Vol. 274, No. 38, pages 7331-27337

D4: Rudolph R., et al., "*In vitro folding of inclusion body proteins*", FASEB Journal (1996), Vol. 10, No. 1, pages 49-56

Annex MD1: additional technical information filed with the statement of grounds of appeal

IX. The appellant's arguments are summarised as follows:

Admittance of the main request
(Article 12(4) RPBA 2007)

The request should be held admissible because the amendments were not complex and were suitable to address the objections raised.

Main (sole) request

Amendments (Article 123(2) EPC)

The new main request addressed issues raised by the examining division and the board. The amendments were directly and unambiguously derivable from the application as filed.

Clarity (Article 84 EPC)

The amendments to claim 1 in step a), specifying the rPvRII sequence, and step b), specifying the expression system as the BL21 (DE3) strain of *E. coli*, addressed objections raised by the examining division under Article 84 EPC.

Inventive step (Article 56 EPC)

The process as claimed enabled the preparation of a stable immunogenic protein having multiple cysteines with a purity of more than 98% and stability of up to two years at low temperatures and six months at room temperature.

The process was industrially feasible and increased yields from 5% to about 20%. As evidenced in Example 1 of the application, the novel washing buffer system resulted in removal of most of the host-cell proteins (see page 16, first paragraph) while the refolding system had been chosen after many trials with different components (see page 17, last paragraph). The other steps described in the examples were common steps in protein production and had no influence on the advantageous effects achieved by the claimed process.

None of the relevant prior-art documents D1 to D4, either alone or in combination, provided a hint or prompt that led to the claimed subject-matter.

Document D1 disclosed working at high cell densities.

Document D2 disclosed that larger proteins with many cysteine residues were problematic and lower folding yields could be expected. Thus, the skilled person would not assume that the system disclosed in document D2 was applicable to the rPvRII protein, which had a high number of cysteine residues.

Document D3 disclosed granzyme K - a protein not containing any cysteine residues at all.

Document D4 disclosed the redox-pair as claimed, but the compositions of the washing and refolding buffers needed adjusting.

Process conditions were not easily adapted. This was apparent from the comparative data in Annex MD1 (provided with the statement of grounds of appeal), which showed that none of the processes described in the prior-art documents D1 to D4 provided the protein in yields achieved by the claimed process as demonstrated by Example 1, Table 2 of the application as filed and Annex MD1.

X. The appellant requested that the examining division's decision be set aside and that the case be remitted to the examining division with the order to grant a patent based on the main request as submitted during oral proceedings.

Reasons for the Decision

Admissibility of the appeal

1. The appeal complies with Articles 106 to 108 and Rule 99 EPC and is therefore admissible.

Admittance of the main request (Article 12(4) RPBA 2007)

2. The main request was filed at the oral proceedings to address objections under Articles 123(2), 84 and 56 EPC raised by the board with regard to the then pending requests. It differs from the request dealt with in the decision under appeal essentially in that the subject-matter of the claim is limited to a process for the expression, purification and refolding of rPvRII, additionally specifying:

- the amino acid sequence of rPvRII (step a)),

- the BL21 (DE3) strain of *E. Coli* as the expression system (step b)),

- that solubilisation is performed using chaotropic agents comprising guanidine hydrochloride at a concentration of 6 to 8 M (step d)),

- that the refolding buffer contains a detergent-dextrin mixture, a co-solvent and a redox pair (step f)),

the detergent used being Triton X-100 at a concentration of 0.1-1.0 mM, a dextrin at a concentration of 5-20 mM, with L-Arginine as a co-solvent being added in a concentration of 0.4-0.8 M and a redox pair comprising L-cysteine - cystamine dihydrochloride.

3. Given that the amendments are not complex and are considered suitable to address the objections raised, the board decided to admit the main request into the proceedings (Article 12(4) RPBA 2007).

Main (sole) request

Added subject-matter (Article 123(2) EPC)

4. As submitted by the appellant, the subject-matter of claim 1 has a basis in the application as filed in the following passages: claims 1 and 2; Table 1 (recombinant *Plasmodium vivax* region II (rPvRII) sequence); page 4, third full paragraph (full designation of rPvRII); claims 4 and 5, and page 11, lines 18 et seq. (washing buffers); page 13, line 7 (solubilisation with chaotropic agent); claim 8 and page 11, last paragraph to page 12, first paragraph (refolding conditions).

The basis for claims 2 and 3 is found in claims 6 and 9 of the application, respectively.

Consequently, the main request complies with the requirements of Article 123(2) EPC.

Clarity (Article 84 EPC)

5. Contrary to the examining division's findings (see section II. above) the board agrees with the appellant that all essential technical features of the claimed process are present in claim 1; they are to be found in step d) (the washing steps) and step f) (the refolding steps).

The main request complies with the requirements of Article 84 EPC.

Novelty (Article 54 EPC)

6. The decision under appeal did not address novelty. The board finds that none of the prior-art documents on file discloses the process according to claim 1.

The main request meets the requirements of Article 54 EPC.

Inventive step (Article 56 EPC)

Closest prior art

7. The board agrees with the examining division that document D1 represents the closest prior art given that it deals with the recombinant production of the protein rPvRII in *E. coli* as well as its isolation and refolding (see abstract and chapters "*Analysis of recombinant PvRII expression*" *and* "*Refolding by rapid dilution method and purification by ion exchange chromatography*" on page 1892, left-hand column of document D1).

Difference between the claimed subject-matter and the closest prior art

8. The claimed process differs from that disclosed in the closest prior art on account of four features:

a) a specific rPvRII protein sequence is provided;

b) before solubilisation, the inclusion bodies undergo washing with a low-salt and subsequently a high-salt buffer (step d) of claim 1);

c) refolding is performed in a buffer containing 0.1-1.0 mM Triton-X-100 as a detergent, 5-30 mM of a dextrin, and a redox-pair comprising L-cysteine - cystamine dihydrochloride (step f) of claim 1); and

d) 0.4-0.8 M L-arginine is used as a co-solvent in the refolding buffer (step f) of claim 1).

Technical effect of said differences and objective problem to be solved

9. On the basis of Example 1 and Table 2 of the application and Annex MD1 as provided with the statement of grounds of appeal, the appellant argued that the technical effect of the different features was a higher yield, in particular in industrial-scale production, so the problem should not be formulated merely as the provision of an alternative process for purifying rPvRII protein.
The issue of whether the problem is to be formulated as an improvement over the disclosure in document D1 can be left open in view of the board's conclusion that the claimed subject-matter is not obvious when the problem

is formulated as the provision of an alternative process for producing rPvRII.

Obviousness

10. The question to be answered when assessing the obviousness of the claimed subject-matter is whether or not, in the light of the closest prior-art document D1, other prior art documents or common general knowledge, the skilled person faced with the problem of providing an alternative process for purifying the rPvRII protein would have modified the process disclosed in document D1 such as to arrive at the claimed process, which, as identified above in point 8., differs from that in document D1 on account of four process features.

11. Document D1 neither teaches nor suggests any of the four different features.

12. In the decision under appeal the examining division took the view that the use of two different washing steps was known from documents D2 and D3, that redox systems for protein refolding were known from documents D3 and D4 and that the skilled person would have adapted the measures disclosed in these documents to the protein in hand by routine experimentation.

Washing buffer

13. Document D2 provides a general protocol for purifying recombinant proteins purified from *E. coli* inclusion bodies, teaching that the pellet fraction (consisting of inclusion bodies) is washed repeatedly with buffers with and without urea and Triton X-100 (the washing conditions are provided in paragraph 1 on page 6.3.8

and the detailed protocol in "*Prepare washed pellets*" on pages 6.3.2 to 6.3.3).

Document D3 discloses purification of recombinant granzyme K *inter alia* by washing *E. coli*-derived inclusion bodies twice with 50 mM Tris-HCl, 60 mM EDTA, 1.5 M NaCl, 6% Triton X-100, pH 7.2, followed by two washing steps in 50 mM Tris-HCl, 60 mM EDTA, pH 7.2.

Thus, neither D2 nor D3 discloses the washing buffer according to claim 1.

Refolding buffer

14. Document D3 refolds the inclusion bodies in 100 volumes of 50 mM Tris-HCl, 0.5 M L-arginine, 20 mM $CaCl_2$, 1 mM EDTA, 0.1 M NaCl, 0.5 mM L-cysteine, pH 8.5, at room temperature (see page 27332, right-hand column, first two lines).

Review article document D4 describes the isolation of inclusion bodies on page 50, right-hand column. The refolding procedure is provided on page 51, right-hand column, which explicitly states that "*the optimum* [refolding] *procedure has to be determined on a case-by-case basis.*" A list of different redox pairs is provided on page 52, right-hand column, third paragraph, together with the statement that "*other low molecular weight thiols* [...] *may be superior for disulfide formation* [remark: compared to reduced and oxidized glutathione as discussed in the sentence before to be commonly used] *depending on the respective inclusion body protein.*"

Thus, neither D3 nor D4 discloses the refolding buffer composition of claim 1.

15. As observed above, none of the documents referred to by the examining division discloses the exact washing and refolding buffer compositions as claimed. Yet in the decision under appeal the examining division was of the view that the skilled person would have arrived at them by mere routine experimentation.

16. The board accepts that the skilled person knew that the conditions in individual process steps had to be adapted to the specific protein being processed when purifying recombinant proteins from inclusion bodies.

17. The purification of the rPvRII protein from inclusion bodies is a multi-step process and many variables and options are available for selecting suitable buffers, excipients and process steps. The board has not seen evidence that, in view of this, mere routine screening and experimentation would lead to the claimed process, as compared with that disclosed in document D1.

The board sees its conclusion confirmed e.g. by this statement in review article D4: "*In vitro folding of inclusion body proteins was considered to be an extremely difficult task. Many frustrations were caused by attempts to transfer folding protocols directly from one folding problem to another.*" (see page 54, right-hand column, paragraph 3).

18. Thus, the board concludes that the skilled person faced with the problem of providing an alternative process for purifying the rPvRII protein would not have modified the process disclosed in document D1 such as to arrive at the claimed process.

19. Hence the subject-matter of claims 1 to 3 involves an inventive step and thus complies with the requirements of Article 56 EPC.

Order

For these reasons it is decided that:

1. The decision under appeal is set aside.

2. The case is remitted to the examining division with the order to grant a patent based on the main request and a description and drawings to be adapted thereto.

The Registrar: The Chair:

I. Aperribay G. Alt

Decision electronically authenticated