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(57) **Abrégé/Abstract:**

The present invention relates to bacteriophage tail proteins and the derivatives and fragments thereof that are capable of binding endotoxins in the absence of bivalent positive ions, especially Ca^{2+} or Mg^{2+} . Further, the present invention relates to methods for depleting endotoxins from solutions and samples using the bacteriophage tail proteins according to the present invention and to a detection method for endotoxins.



Abstract

The present invention relates to bacteriophage tail proteins and the derivatives and fragments thereof that are capable of binding endotoxins in the absence of bivalent positive ions, especially Ca^{2+} or Mg^{2+} . Further, the present invention relates to methods for depleting endotoxins from solutions and samples using the bacteriophage tail proteins according to the present invention and to a detection method for endotoxins.

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Method for detecting and removing endotoxin

The present invention relates to bacteriophage tail proteins and the derivatives and fragments thereof that are capable of binding endotoxins in the absence of bivalent positive ions, especially Ca^{2+} or Mg^{2+} . Further, the present invention relates to methods for depleting endotoxins from solutions and samples using the bacteriophage tail proteins according to the present invention and to a detection method for endotoxins.

10 Bacteriophages recognize structures (membrane proteins, sugar molecules etc.) on the surface of their host bacteria by corresponding proteins, which bacteriophages have on their surface. Some bacteriophages have only one type of recognition protein, e.g. salmonella phage P22, others at least two or more. The recognition proteins may have enzymatic activity, as phage P22 (Seckler, J. Struct. Biol. 1998; 122(1-2):216-222), or
15 may not have enzymatic activity. Enzymatic activity means, that these proteins, e.g. the P22 tail spike protein, are able to hydrolyze the receptor molecule, that they recognize, i.e. at p22 the salmonella O-antigen. The most known bacteriophage having two recognition proteins is for E.coli the specific phage T4. This phage has long and short tail fibers. The long tail fibers conduce the specific recognition of its host E.coli by the membrane
20 protein OmpC or by lipopolysaccharide for E.coli B. While the long tail fibers of the phages T4, T2 and K3 bind specifically to OmpC and lipopolysaccharide of E.coli B (T4), respectively, OmpF (T2; Hantke K., Mol Gen Genet. 1978; 164 (2):131-135) and OmpA (K3; Hancock RE, Reeves P., J Bacteriol. 1975;121(3):983-993; Riede I, Eschbach ML, Henning U., Mol Gen Genet. 1984; 195(1-2):144-152), the short tail fibers are located at
25 the bottom side of the phage and are not involved in the host specificity, but replaceable between T4, T2 and K3 phages (Riede, Mol Gen Genet. 1987;206(1):110-115). Only after the binding of at least three long tail fibers, the short tail fibers are folded out of the basis plate and are responsible for irreversible binding of the T4 phage to the E.coli hosts (Leiman et al., Cell Mol Life Sci. 2003;60(11):2356-2370). These short tail fiber proteins
30 bind, as shown for phage T4 (WO2004/001418), in the core region of the lipopolysaccharide and thus are qualified for recognizing and binding endotoxin.

Endotoxins (ET) describe a family of lipopolysaccharides, which form the outer cell membrane of gram-negative bacteria together with proteins and phospholipids. Endotoxins

only occur in this bacteria group and play an important role at the organization, stability and barrier function of the outer membrane. Numerous bacteriophages use endotoxin and general lipopolysaccharides, respectively, for specific recognition of their host bacteria.

5 Endotoxins are biomolecules which may be found in practically all aqueous solutions without corresponding precautionary measures. Endotoxins effect on human and animals highly pyrogenically, so they cause fever response and are able to result in a sepsis, a heavy dysfunction of the immune system involving a high mortality rate. Therefore contamination with endotoxin, e.g. at the production of proteins for medical or
10 pharmaceutical use, have to be detected exactly and be removed consequently. Endotoxin presents a problem by genetically produced pharmaceuticals, gentherapeutic agents or substances, which are injected into humans or animals (e.g. veterinary treatment or in animal tests). However, not only for medical, but also for research applications, such as transfection experiments of mammalian cells, an inhibition or decrease, respectively, of
15 the transfection efficiency by endotoxin may be found.

All endotoxin variations consist of a heteropolysaccharide, that is covalent bound to lipid A (Holst, O., 1999, In: Endotoxin in health and disease (Brade, H. et al; eds.), Marcel Dekker Inc. New York)). Lipid A anchors endotoxin in the outer bacteria membrane. The
20 heteropolysaccharide, consisting of a core oligosaccharide and the O-antigen, points to the ambient solution and determines the serological identity of the bacterium. The O-antigen consists of repetitive oligosaccharide units, whose composition is specific for each strain (see Holst et al., supra). Characteristic blocks of the core oligosaccharide are 2-keto-3-deoxyoctonic acid (KDO) and L-glycero-D-manno-heptose (Hep).

25

The most conservative part of different genera of endotoxin is the Lipid A. The inner heart region is related conserved as lipid A, while the outer core region already has a higher variation. The inner heart region, KDO and lipid A carry several phosphate groups as substitutes themselves and are consequently responsible for the negative charge of
30 endotoxin. Furthermore, the phosphate groups of Lipid A and the core region may be substituted with arabinose, ethanolamine and phosphate variably. Single saccharide building blocks of the O-antigen are acetylated, sialylated or glycolysated. The O-antigen varies moreover concerning the amount of repetitive units, wherefore the endotoxin population of each bacterium has a certain heterogeneity (Palva E.T. and Makela P.H., Eur

J Biochem. 1980;107(1):137-43; Goldman R.C. and Leive L., Eur J Biochem. 1980;107(1):145-53).

To be able to use proteins within clinical studies, the European and American
5 pharmacopoeia demand, that the proteins under-run certain limit values of endotoxin load
(e.g. immune serum globulin $\leq 0,91$ EU/ml, this corresponds to ≤ 5 EU/kg body weight &
hour (dose rate = EU/kg * h); EU = endotoxin unit; FDA (Food and Drug Administration):
Guideline on Validation of LAL as End Product). In case a drug and therein-containing
10 proteins, respectively, have a too high endotoxin load, it is possible that this induces the
death of the patient. The misdirected immune defense damages the patient by an over-
reaction. This may induce tissue inflammation, decrease in blood pressure, tachycardia,
thrombosis culminating in septic shock and multiple organ failure. Already a long running
exposition of endotoxin in picogram quantities may induce chronic side effects e.g. low
immunity, septic symptoms etc.. Within the substance production, it is tried to deplete and
15 remove, respectively, endotoxin as far as possible, in particular in processes of "Good
Manufacturing Practice" (GMP) conditions. However, the removal of endotoxin on
proteins, polysaccharides and DNA is problematically. In particular, great problems exist
on proteins, because of whose intrinsic properties as charge state or hydrophobicity, which
almost inhibit endotoxin removal and may lead to great losses of products, respectively,
20 during the removal process.

Furthermore, the endotoxin detection as well as the removal is affected by the
environment, since factors e.g. ion composition, pH-value, temperature or the presence of
other substances may influence the interaction of a ligand with endotoxin rigorously.
25 Thereby it must be considered, that the interaction of ligands may be carried out with
different structure parts of the endotoxins as the hydrophobic Lipid A or the hydrophilic
polysaccharide part. According to this, normally these interactions depend on ionic or
hydrophobic forces, which are affected differently by the composition of the solution. The
polysaccharide structure of endotoxins is stabilized by bivalent positive ions as calcium or
30 magnesium (Galanos C. and Lüderitz O., Eur. J. Biochem. 1975; 54:603-610). These ions
are also able to interfere with ligands ("bridging-effect").

In general, there are a number of methods for depleting and removing endotoxin,
respectively, from biological solutions. However particularly for proteins, there are no

general applicable standard methods so far. The used methods are adapted to the special properties of the protein and its corresponding production process in each case. There are different opportunities for depleting endotoxins, wherein each of these methods has specific advantages and disadvantages.

5

The ultra filtration (Petsch, D. & Anspach, F.B., 2000, J. Biotechnol. 76, 97-119 and references therein) is used for depleting endotoxin from water and solutions with low-molecular substances as salts, sugar and antibiotics. However, it is not qualified for high-molecular proteins or DNA.

10

The two-phase extraction (e.g. WO 01/66718, Merck) should separate water-soluble proteins and DNA from endotoxin, but it involved detergent residues in the purified product. However, the method is time-consuming because of repeating the purification process for several times.

15

Likewise, an anion exchanger process (DEAE) (e.g. US 5,990,301, Qiagen; WO 94/14837, Enzon; EP0592989, Braun Melsungen) is used for depleting endotoxins from DNA and acidic proteins, but it requires a low ionic strength (<50 mM NaCl) and leads to a protein co-adsorption of acidic proteins. For alkaline proteins cation-exchanger are used, which partly are combined with detergents (e.g. US 2002/0147315 A1).

20

Cationic peptides are used for removing endotoxin in EP 0232754 B1 (Commonwealth Biotechnologies).

25 In addition, hydrophilic matrices are used as a combination of dextran and N',N'-methylenebisacrylamide (US 5,917,022).

Hydrophobic chromatography methods are used in WO94/14837 (Enzon).

30 The affinity adsorption (e.g. polymyxin B, histamine, histidine, poly-L-lysine, polyethylenimine) e.g. GB 2,192,633 (Hammersmith Hospital), US2002/0130082 A1 (Tokodoro), US5,510,242 or WO95/025117 (GMBF) is a further method for depleting

endotoxins from DNA and proteins (e.g. BSA, myoglobin, gamma globulin, cytochrome C), but it is toxic in the case of polymyxin B and may lead to a co-adsorption of proteins at low ionic strength.

- 5 Following methods describe a removal of endotoxin by means of metal affinity chromatography (US6,365,147; US6,942,802; WO02/083710, American Cyanide).

In addition, LPS-binding proteins or peptides or derivates thereof are used for specific binding of endotoxin (US6,376,462, Xoma Corp.; US6,384,188, Dana Faber Cancer
10 Institute; WO95/005393, Morphosys; WO95/008560, Centocor; WO95/025117, Scripps).

Further on, the immune-affinity chromatography is used, wherein the specificity for certain endotoxins can only be achieved by expensive antibodies against core oligosaccharide (US 5,179,018, Centocor; WO 00/08463, Bioserv; EP0074240, Gaffin).

15

Further, the S3 delta peptide (WO 01/27289) of the factor C (a component of the LAL-test) (WO 99/15676 both: National University of Singapore) is used for proteins (e.g. BSA, chymotrypsinogene), however this method has a low efficiency at high ionic strength and high production costs come along (production of insect cell cultures).

20

Furthermore the endotoxin neutralizing protein (ENP) from *Limulus polyphemus*, that also binds specifically to endotoxin (e.g. US5,747,455; US5,627,266) or the LPS binding protein of the horseshoe crab (US5,760,177) is used for depleting endotoxins. The recovery of this protein from the horseshoe crab or recombinant from *saccharomyces* is
25 also time-consuming and cost-intensive.

A further method for removing endotoxins from a sample is described in the WO2004/001418. Thereby endotoxins are bound to a carrier immobilized with bacteriophage tail proteins and are so separated from the sample. For an efficient
30 separation, bivalent ions are necessary by what the method cannot be carried out with industrial relevant buffers e.g. phosphate or citrate buffers or in the presence of chelators as EDTA or EGTA.

Essentially three methods exist for protein solutions adapted to the properties of the target proteins in application in pharmaceutical industry:

- anion exchange chromatography

5 • reserved-phase chromatography; This has the disadvantage, that it is not suitable for all proteins similarly and for hydrophobic proteins particularly problematically. Furthermore, this method is very time-intensive and proteins are normally denaturated under the conditions of the reserved-phase chromatography, so that they have to be renaturated afterwards time-consuming and often with a high
10 material loss

- RemTox (Fa. Milipore): This method has the disadvantage that beside a very long incubation time the unspecific binding fraction is high and the recovery of proteins is often not sufficiently.

15 A rough depletion of endotoxin from proteins to a value of up to 10 EU/ml is possible in numerous cases with the existing methods. However, still the remaining concentrations of endotoxin affect toxically. Therefore, a further depletion (i.e. precision purification) is demanded and dependent, respectively, on the protein doses in the medical application. The European pharmacopoeia, the USP (United States Pharmacopeial Convention) and the
20 FDA (Food and Drug Administration) specify the limit values for medical application bindingly (e.g. 5 EU/kg body weight and hour for intravenous applications). However, the precision purification is often not warranted sufficiently with the present methods. The standard methods have relevant disadvantages and are often not applicable for certain proteins or only with a relevant loss of the target protein.

25

Further, in view of industrial applications it have to be considered, that only buffer substances as phosphate, citrate, borate, carbonate or acetate as cheap as possible are used for reasons of economy. Therefore, the interaction of ligands with endotoxins should not be interfered by these buffers. For binding reactions needing calcium, in particular buffers
30 or additives are problematically, which coordinate calcium as EDTA, EGTA or citrate. In addition, buffers whose salts build insoluble or hardly soluble precipitations with calcium are problematically. For example, calcium phosphate precipitates so there is only a low concentration of free calcium in phosphate buffers.

Beside of depleting and removing endotoxin, respectively, the endotoxin detection in samples, solutions and pharmaceutical preparation plays an important role. Currently six detection methods are described for endotoxin in biologic solutions, wherein only the first two methods are accredited from the FDA. The EAA (endotoxin activity assay) is accredited from the TPD (Therapeutic Product Directorate of Canada) and from the FDA under certain conditions (high risk for sepsis at intensive patients) also. 1. "Rabbit Pyrogen Testing": A method in which an endotoxin solution is injected to a living rabbit to cause an immune reaction. This immune response caused by endotoxin is verified by fever. 2. Clearly better to standardize is the "Limulus Amoebocyte Lysate (LAL)" – test, which is currently the most applied test (Cambrex-BioWhittaker, Inc., Charles River, Inc., Associates of Cape Cod, Inc., all USA). For this method, an enzyme cascade is induced in the blood of the horseshoe crab (*Limulus polyphemus*) after the contact of endotoxin. The existence of endotoxin can be measured by four different methods (gel-clot, turbidimetric, colorimetric and chromogenic assay). 3. The InVitro Pyrogene test based on the detection of interleukine-1 β in human blood, which is involved in the induction of fever. The test consists of an incubation step of human blood with the examining solution and the following detection of interleukins by antibodies. 4. A similar method is the detection of the induction of prostaglandine (PGE2) in rabbit blood after the contact with endotoxin (Ochiai et al., Microbiol. Immunol., 2003, 47, 585-590). 5. A further possibility is the application of a special cell culture systems (Sterogene Inc., USA) with which the activation of monocytes is pursued by the formation of certain cytokins. 6. The EAA (endotoxin activity assay) by the company Spectral Diagnostics, Inc., Canada is also a blood test. Endotoxin reacts with antibodies, wherein the signal is enforced and detected as chemiluminescence after the complement activation in the patient owned neutrophiles by means of a zymosans.

However, the both first named methods are very expensive and not at least critical for nature conservation reasons because of the high demand of test animals and blood of the very rare horseshoe crab, respectively. In fact, the LAL-test is able to be miniaturized and automated but it has massive disadvantages at the application. It is labor-intensive, requires special trained staff, relative long incubation times, relative big sample volumes and expensive reagents. A onetime opened LAL-solution has to be processed and used up directly, because the components aggregate within a few hours because of low stability. Bivalent ions have to be present in the application of the test, the pH-value is relatively

limited (pH 6-7,5) and present glucans often interfere the test. Endotoxin is often masked, i.e. it is e.g. not recognized, if it is bound to proteins. The InVitro Pyrogen test requires as fresh human blood as possible and is relative time-intensive, because the production of the interleukins requires 10 to 24 hours. The main advantage of this method is that also other pyrogens are detected beside endotoxins. This test is primarily intended for replacement of the "Rabbit Pyrogen test". For all test methods, trained stuff is required and the methods are sensitive for interference, because e.g. it is possible that the immune system of rabbits reacts differently at the same dose of endotoxin. The cell culture method of the company Sterogene is also, as all cell culture methods, very complex and has problems with the standardization. If the different methods for detecting endotoxin are compared, the results often differ from each other, i.e. different endotoxins are not recognized by different test components in the same way. Altogether, it can be fixed, that no easy manageable economic method exists for detecting endotoxin and that the currently used methods have numerous disadvantages.

Therefore, the object of this invention is to provide instruments and methods for removing and detecting endotoxins out and in solutions independent of the content of bivalent positive ions.

The problem of the invention is solved by the subject matter of the claims.

The following figures illustrate the invention.

Figure 1 shows the result of the endotoxin removal by T4p12-sepharose (A), N-Strep-Miro2p12-sepharose (B) and N-Strep-Aeh1p12-sepharose (C) from "calcium containing" (with calcium) and "calcium free" (without calcium) buffers. The black bars show respectively the applied endotoxin amount and the dashed bars the endotoxin amount after the run of the solution by the corresponding sepharose. The endotoxin amounts were measured by the LAL-test (kinetic-chromogenic LAL-test, Cambrex) and are specified in endotoxin units (EU). At first, the bacteriophage tail proteins were immobilized on NHS-activated sepharose (Amersham Biosciences) and afterwards chromatography columns were cast with these coupled sepharose material. Columns were cast with volumes of 1 ml (T4p12, N-Strep-miro2p12) and 200 µl (N-Strep-Aeh1p12). As column bodies 1 ml polypropylene columns (Qiagen) and Handee Mini Spin Columns (200 µL, Pierce) were used. At first, the columns were equilibrated with running buffer (6-times column

volume); the sample was applied and afterwards washed with 3-times column volume. The endotoxin amounts in the application (black bars) and in the flow-through (dashed bars) are represented respectively side by side. The tests were carried out in “calcium containing” and “calcium free” buffer. The “calcium containing” buffer consisted of 20 mM Hepes, 150 mM NaCl, 0.1 mM CaCl_2 , pH 7.5 and the “calcium free” buffer consisted of 20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7.5. With T4p12 sepharose it was only possible to remove endotoxin with the “calcium containing” buffer from the solution, while calcium had no influence of the endotoxin removal with N-Strep-Miro2p12 and N-Strep-Aeh1p12 sepharose.

Figure 2 A-C shows the result of the endotoxin removal from a BSA solution by chromatography columns with immobilized Miro2p12 in comparison to columns of polymyxin and T4p12. The running buffer (20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7.5) contained 2 mM EDTA and therefore only a low concentration of free calcium ($< 1 \mu\text{M}$; Donald Bers, Chris Patton, Richard Nuccitelli, *Methods in Cell Biology*, Vol. 40; A practical guide to the study of Ca in living cells; chapter1: A practical guide to the preparation of Ca Buffers, Academic Press, 1/94). The chromatography columns contained each 2 ml column material. Before the application of the sample (F0), 1 ml flow-through of running buffer was collected (F1). Afterwards, each 1 ml of a BSA-solution (1.2 mg/ml), studded with approximately 2000 EU/ml, was applied onto each column and two further fractions were collected with 4 ml and 3 ml (F2 and F3). In Fig.2(A) the amounts of endotoxin and in Fig 2(B) the amounts of BSA in the application and in the fractions F1-F3 are represented of the polymyxin B-column (spotted bars), the T4p12 column (dashed bars) and the Miro2p12 column (filled bars). In Fig. 2(C) the percental endotoxin removal (filled bars) and the protein recovery (dashed bars) is shown calculated for the second fraction. By T4p12 it was not possible to remove endotoxin under these buffer conditions, while it was possible to remove 96% of endotoxin by the polymyxin B column and 99,6% by the Miro2p12 column. The protein recovery was 67% for polymyxin B and 92% for Miro2p12. The percental endotoxin removal shows how much endotoxin, available at the beginning of the experiment, was removed by the treatment with Miro2p12 or other proteins. The protein recovery informs how much of the applied protein is still in the sample after the endotoxin removal and is unspecifically removed, respectively, by the endotoxin removal.

Figure 3 A-C shows the result of the endotoxin removal from a BSA solution by

chromatography columns immobilized with Miro2p12 in comparison to columns of polymyxin B and T4p12. As running buffer the physiologic important PBS buffer (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4) was used in this experiment. The chromatography columns contained each 2 ml column material. Before the application of the sample (Fraction F0), 1 ml flow-through of the running buffer was collected (F1). Afterwards each 1 ml of a BSA solution (1.2 mg/ml), studded with approximately 1800 EU/ml, was applied onto each column and two further fractions were collected with 4 ml and 3 ml (F2 und F3). In Fig.3(A) the endotoxin amounts and in Fig 3(B) the BSA amounts in the application and in the fractions F1-F3 are represented of the polymyxin B-column (spotted bars), the T4p12 column (dashed bars) and the Miro2p12 column (filled bars). In Fig. 3(C) the percental endotoxin removal (filled bars) and the protein recovery (dashed bars) is shown calculated for the second fraction. By polymyxin B it was possible to remove 97% of endotoxin, by T4p12 74% and by Miro2p12 99.7%. The protein recovery was 61% for polymyxin B, 99.4% for T4p12 and 99.8% for Miro2p12.

Figure 4 A-C shows the result of the endotoxin removal from a BSA solution by chromatography columns immobilized with Miro2p12 in comparison to columns with polymyxin B and T4p12. A citrate buffer (20 mM citrate, 150 mM NaCl, pH 7.0) was used, which is able to bind calcium ions. The chromatography columns contained each 1.5 ml column material. Before the application of the sample (Fraction F0), 1 ml flow-through of the running buffer was collected (F1). Afterwards each 1 ml of a BSA solution (0.96 mg/ml), studded with approximately 750 EU/ml, was applied onto each column and two further fractions were collected with 4 ml and 3 ml (F2 and F3). In Fig.4(A) the endotoxin amounts and in Fig 4(B) the BSA amounts in the application and in the fractions F1-F3 are represented of the polymyxin B-column (spotted bars), the T4p12 column (dashed bars) and the Miro2p12 column (filled bars). In Fig. 4(C) the percental endotoxin removal (filled bars) and the protein recovery (dashed bars) is shown calculated for the second fraction. By polymyxin B it was possible to remove 96.2% of endotoxin, by T4p12 0% and by Miro2p12 99.5%. The protein recovery was 78.3% for polymyxin B, 97.8% for T4p12 and 95.4% for Miro2p12.

Figure 5 shows the result of the endotoxin detection by the binding of T4p12 to immobilized endotoxin (Miro2p12-endotoxin-T4p12 sandwich). The bacteriophage tail protein Miro2p12 was covalent immobilized (3.4 mg protein/ml column material) to the

column material (0.5 ml). A sample, containing endotoxin of *E.coli* O55:B5 (10^6 EU/ml), was applied onto the column and bound by Miro2p12 (+LPS, black bars). A control column was equilibrated with the sample without endotoxin (-LPS, dashed bars). The amount of the bacteriophage tail protein p12 was plotted against the fractions of the chromatography run. Each bar shows the averages detected in two parallel chromatography runs. The first bar pair (F0) shows the applied amount of T4p12 and the second the fraction 1 (F1), a control fraction before the application of T4p12 onto the column. The arrow marks the application of p12 onto the column. The fractions F2–F5 were collected after the application. The amount of p12 (stated in mg) was detected by absorption measurement at 280 nm. The fraction volume was 1 ml for fractions F1-F4 and 2 ml for fraction F5. The dissolution of bound T4p12 protein in fraction F5 was affected by addition of 2 mM EDTA to the running buffer (20 mM Hepes, 150 mM NaCl, 0.1 mM CaCl_2 , pH 7.5). The bacteriophage tail protein T4p12 was retained at the columns prior loaded with endotoxin, while it elutes without delay through the columns containing no endotoxin.

Figure 6 shows the result of the binding of Miro2p12 by endotoxin arranged to T4p12. A CM-5 chip of company Biacore was used with covalent immobilized T4p12 (EDC/NHS-coupling). At first, endotoxin (of *E. coli* O55:B5; 1 mg/ml) was injected, which binds to T4p12 as shown by the increase of the resonance signal. Afterwards Miro2p12 (0.02 mg/ml) was injected, which also binds as shown by the increase of the resonance signal. For ending the experiment, the endotoxin-T4p12 binding was again released by addition of EDTA. The injection phases are indicated by the bars. As running buffer 20 mM Hepes, 150 mM NaCl, 0.1 mM CaCl_2 was used. The second unloaded cell of the chip was used as a reference cell. The curve shows the difference between measure and reference cell. RU means resonance units; s means seconds.

Figure 7 shows the result of the endotoxin removal from human serum by N-Strep-Miro2p12 immobilized to sepharose. Each 1 ml of human serum, studded with endotoxin of *E.coli* O55:B5 (F0), was applied onto two N-Strep-Miro2p12-sepharose columns (column volume: 1 ml, column1 = black bars, column2 = dashed bars) and was collected in fractions of 4 and 3 ml, respectively, (F2, F3) after the columns. The arrow marks the application of sample. Before the application, the column was washed with 1 ml running buffer to be sure, that the column is not contaminated by endotoxin. The running buffer was composed of the following: 20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7.5. It

was possible to remove 90 % of the applied endotoxins from the serum, i.e. to be retained on the column.

Figure 8 shows the result of the endotoxin removal by Aeh1p12 immobilized to sepharose (2.5 mg Aeh1p12 per 1 ml sepharose) in comparison to sepharose, which is loaded with ethanolamine instead of Aeh1p12 to saturate the reactive groups of the activated sepharose. The experiment was carried out with and without calcium in the running buffer to determine the influence of calcium to the endotoxin removal. The running buffers were composed of as following: black bars (+ Ca): 20 mM Hepes, 150 mM NaCl, 0.1 mM CaCl₂, pH 7.5. Dashed bars (- Ca): 20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7.5. Each 0.2 ml columns were cast und washed with 2 ml 0.5% sodium deoxycholate, 15 ml water and 2 ml running buffer before the experiment. Afterwards 0.2 ml lipopolysaccharide of *E.coli* O55:B5 (approximately 1000 EU/ml), solved in their respective running buffer, were applied. Said sample was incubated for 30 min at room temperature, was allowed to run through the column and was washed with 0.6 ml and 1 ml running puffer. The endotoxin content of the fractions was determined via LAL-test and the percental endotoxin removal was calculated by the amounts of endotoxin before and after the column. It was possible to remove significant more endotoxin from the solution by Aeh1p12 (88% and 84%) as by the control column (25% and 39%) containing no protein. Free calcium is not necessary for binding endotoxin to Aeh1p12, because the endotoxin removal was similarly with calcium (88%) and without calcium (84%).

Figure 9 shows the result of the endotoxin removal by Effe04p12. For this, the protein was coupled covalent to sepharose and afterwards incubated with an endotoxin solution, which was again separated from sepharose by centrifugation. The endotoxin removal was carried out in calcium free buffer (20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7,5). At first 100 µL Effe04p12-sepharose was washed with 1 ml endotoxin free buffer (F1) and afterwards 100 µL endotoxin solution (lipopolysaccharide of *E.coli* O55:B5) was applied onto said Effe04p12 sepharose. The sepharose was incubated for 30 min with the endotoxin solution and mixed for several times in doing so. Afterwards the solution was separated from sepharose by a Mini-Spin column and washed with 200 µL buffer (F2). Finally, the solution was washed another two times with each 200 µL (F3 and F4). The endotoxin content in the application (F0) and the fractions was detected via LAL-test and out of it, the percental endotoxin removal was calculated to 92.5%.

Figure 10 shows the core-sugar structures of *E.coli* mutants, which were used for binding investigations with N-Strep-Miro2p12. Hep= heptose, Kdo = 2-keto-3-deoxy-octonate, Glc = glucose, Gal = galactose, GlcNAc = N-acetylglucosamine.

- 5 Figure 11 shows the sequence comparison on the level of amino acids between bacteriophage tail proteins. The sequence comparison was carried out with Clustal V (1.81) under following settings, wherein the sequences of T4p12, T2p12 and K3p12 were first aligned with the “Multiple Alignment Mode”: Alignment parameter:

10

Pairwise Parameters:

Pairwise Alingmentsd: Slow-Accurate

Gap Opening [0-100]: 10

Gap Extension [0-100]: 0.1

- 15 Protein Weight Matrix: Gonnet 250

Multiple Parameters

Gap Opening[0-100]: 10

Gap Extension [0-100] 0.2

- 20 Delay Divergent Sequences (%): 30

Protein Weight Matrix: Gonnet Series

Protein Gap Parameters

Residue-specific Penalties: ON

- 25 Hydrophilic Penalties: ON

Hydrophilic Residues: GPSNDQEKR

Gap Separation Distance [0-100]: 4

End Gap Separation: OFF

- 30 Quality – Column Score Parameters:

Score Plot Scale: 5

Residue Exception Cutoff: 5

Protein Weight Matrix: Gonnet PAM 250

35

The sequences of the phages can be located in the protein sequence database of the NCBI for the alignment and they are new isolated proteins by the inventors, respectively, and sequences thereof. 44RR2.8t, Acc.No: AAQ81466; RB49, Acc.No: AAQ15392; T2, Acc.No: CAA39905; T4, Acc.No: AAD42417; PP01, Acc.No: BAD20635; RB69, Acc.No: AAP76072; Aeh1, Acc.No: AAQ17871; KVP40, Acc.No: AAQ64417; AR1, Acc.No: AAN03609. The corresponding proteins of the phages PHG31 and RB43 can be

40

found under <http://phage.bioc.tulane.edu/>; the protein of the phage K3 is described in Burda M.R., Hindenach I., Miller S., Biol. Chem. (2000) 381, 225-258.

Figure 12 shows the result of the endotoxin detection by the formation of a Miro2p12-endotoxin-Miro2p12-sandwich in a column chromatography method. Four columns were used (volume 1 ml), in which Miro2p12 was covalent coupled to sepharose (5 mg protein/ml column material), as described in example 5. Two of these were loaded with LPS of *E.coli* O55:B5 (10^6 EU in 1 ml PLS buffer, 10 mM sodium phosphate, 70 mM NaCl, pH 7.4) (+ET, black bars) and two were used as controls (-ET, white bars). As running buffer 10 mM sodium phosphate, 80 mM NaCl, pH 7.4 was used. Miro2p12 was applied onto all columns (each 600 μ L of a solution with 0.1 mg/ml protein). The amount of the applied and eluted Miro2p12, respectively, was detected by absorption at 280 nm. The amount of bacteriophage tail protein Miro2p12 was plotted against the fractions of the chromatography run. Fraction 3 (F3) shows the flow-through of Miro2p12 after the application (F0), so all bacteriophage tail protein, which was not retained by the column, fraction 4 (F4) is a wash fraction. After the washing, the regeneration buffer RB (10 mM sodium phosphate, 500 mM NaCl, pH 7.4) was added (see arrow direction), which released Miro2p12 bound to endotoxin from the column. Following, fractions 5 and 6 were collected. Fraction F3 has a volume of 0.6 ml, all other fractions have a volume of 1 ml. As control the application onto the column (F0) is represented with the total amount of Miro2p12. It can be seen, that Miro2p12 is retained in the columns immobilized with endotoxins beforehand, while only a small amount of Miro2p12 is unspecifically bound to the control columns without endotoxin.

Figure 13 shows the result of the direct detection of immobilized endotoxin by biotin labeled Miro2p12. LPS of *E.coli* O55:B5 (3 μ g/ml) was immobilized to PolySorp-plates (Nunc) by adsorption (18h at room temperature in PBS buffer, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4). Afterwards the microtiter plates were blocked with casein (0.05% in PBS, 1.5 h at RT) and washed with PBS buffer for one time. Control plates were not incubated with endotoxin but only blocked with Casein. Each 200 μ l Miro2p12 labeled with biotin (Miro2p12-bio) in 50 mM Tris, pH 8, 0.05% Casein, 0.05% Tween20 was added in raising concentration (white bars: plates without

ET, black bars: plates with ET, protein concentration as described). The detection of endotoxin bound to Miro2p12 labeled with biotin was carried out by an absorption measurement at 405 nm after the binding of a conjugate of Streptavidin with alkaline phosphatase (Amersham Biosciences) during the addition of pNPP (para-nitrophenylphosphate) in a concentration of 0.8 mg/ml as a chromogenic substrate. Miro2p12 labeled with biotin binds in a concentration dependent form to the microtiter plates, which were coated with endotoxin previously.

Figure 14 shows the result of binding of endotoxin labeled with FITC at a surface, which were previously coated with Miro2p12. The detection of endotoxin bound to bacteriophage tail protein is carried out in a FITC specific ELISA. Miro2p12 (each 200 μ l with 5 μ g/ml protein) was adsorbed to a MaxiSorp plate (Nunc) (16 h at room temperature in PBS, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4). Afterwards the microtiter plates were blocked with casein (0.05% casein in PBS, 1.5 h at room temperature, each 200 μ L per well) and washed with PBS buffer for one time. Control plates were not incubated with Miro2p12, but only blocked with Casein. Each 100 μ L of FITC labeled LPS of E.coli O55:B5 (Sigma) in PBS were added in a raising concentration (white bars: plates without Miro2p12, black bars: plates with Miro2p12). The detection of the fluorescence labeled LPS bound to Miro2p12 was carried out by the binding of FITC specific antibodies (0.5 μ g/ml, Zymed) in a first step and a secondary antibody, which was conjugated to an alkaline phosphatase (1 μ g/ml, Pierce). The quantification was carried out by a fluorescence measurement of the reaction products of a fluorescent alkaline phosphatase substrate (methylumbelliferylphosphate; Sigma) with 0.1 mg/ml methylumbelliferylphosphate in 50 mM Tris, pH 8 (excitation at 375 nm; emission at 465 nm). Endotoxin labeled with fluorescence binds in a concentration dependent form to the microtiter plates, which were previously coated with Miro2p12.

The term “endotoxin depletion” or “endotoxin removal” as used herein means entire or partial endotoxin removal from the sample material.

The term “sample material” or “sample” as used herein comprises all kinds of solutions, in which endotoxin should be detected or from which endotoxins should be removed.

Exemplary for samples is the following listing: water, aqueous solutions and mixtures of water and organic solvents, blood, blood products, plasma, serum, urine, media, protein solutions, water-ethanol mixtures, food. Also solutions, in which no aqueous solid substances for investigation or for isolation are solved, are further comprised such as protein, DNA, RNA, sugar, salts, drugs, vaccines, food, organic or inorganic chemicals (e.g. NaCl, MgCl₂, purine, pyrimidine, etc.).

The term “endotoxin” as used herein describes bacterial lipopolysaccharide (LPS), which is a component of the outer membrane of gram-negative bacteria.

The term “calcium independent bacteriophage tail protein” as used herein describes such proteins, which can be found in bacteriophages and which are able to bind endotoxin independently of the presence of bivalent positive ions such as Ca²⁺ or Mg²⁺. Usually such proteins are located in the tail of the bacteriophage, but they can also be located on the head of the bacteriophage or at bacteriophages, having no tail, at the normal casing of the bacteriophage. The term bacteriophage tail protein comprises as well short as long bacteriophage tail proteins. So bacteriophages with a basis plate (e.g. myoviridae like T4-similar phages) are able to have different bacteriophage tail proteins, so called long and short bacteriophage tail proteins, which also have different specificity for structures of bacteria membranes. Therefore the term “bacteriophage tail protein” comprises not only the endotoxin binding bacteriophage tail proteins but also the endotoxin binding bacteriophage head proteins and bacteriophage casing proteins. As a result calcium dependent bacteriophage tail proteins are such, which are only able to bind endotoxins in the present of bivalent positive ions as Ca²⁺ or Mg²⁺.

The term “unspecific immobilization” or “undirected immobilization” as used herein means that the coupling of a protein to a matrix occurs by protein residues (e.g. primary amines), which are spread over the total protein surface. The selection of the used group of the single protein molecules for coupling is randomly.

The term “directed immobilization” as used herein means, that the coupling occurs by amino acid residues or other residues (e.g. glycosylation of the protein), their position in

the protein (e.g. N- or C-terminal) is known. The selection of the group for coupling occurs by the selection of suitable reaction partners/linker, which prefer to react with these residues (e.g. coupling of sulfhydryl residues to iodoacetate residues; iodoacetate reacts thousand times faster with sulfhydryl residues as with amino residues).

5

The term “surface” or “carrier” as used herein comprises all materials to which a coupling or adhesion of a protein molecule is possible, e.g. glass surfaces, chromatography materials, e.g. agarose or sepharose, plastic surfaces, e.g. polystyrene or polypropylene, filter materials, e.g. cellulose.

10

The present invention relates to bacteriophage tail proteins and derivatives and fragments thereof, which are able to bind endotoxin independently of the concentration of bivalent positive ions, in particular in the absence of bivalent positive ions, in particular Ca^{2+} and/or Mg^{2+} .

15

In the difference to so called calcium-dependent bacteriophage tail proteins, as such, which are only able to bind endotoxin in the present of bivalent positive ions, in particular Ca^{2+} and/or Mg^{2+} , said calcium-independent bacteriophage tail proteins allow the endotoxin detection in and the endotoxin removal from samples, which contain no bivalent

20

positive ions, in particular Ca^{2+} and/or Mg^{2+} .

The bacteriophage tail proteins according to the present invention are also called in the following as “calcium-independent bacteriophage tail proteins”. Preferred are short tail proteins. Further bacteriophage tail proteins of the family of myoviridae are preferred, in particular of the group of pseudo-T-even, schizo-T-even or T-even phages. Further bacteriophage tail proteins are preferred, which bind to 2-keto-3-deoxyoctonic acid (Kdo) of endotoxins. Further bacteriophage tail proteins are preferred, which are available as trimers and are resistant against sodium dodecyl sulfate (SDS).

25

30

The present invention relates in particular to bacteriophage tail proteins according to SEQ ID NO:2 (Miro1p12), SEQ ID NO:4 (Miro2p12) and SEQ ID NO:6 (Effe04p12) as well as the DNA sequences according to SEQ ID NO: 1 (Miro1p12), SEQ ID NO:3

(Miro2p12) and SEQ ID NO:5 (Effe04p12) encoding the bacteriophage tail proteins according to the invention. The present invention further relates to modified bacteriophage tail proteins and the DNA sequences encoding modified bacteriophage tail proteins according to the present invention. Modified bacteriophage tail proteins have e.g. a His-tag or a Strep-tag to ease the isolation of the proteins after a recombinant production in bacteria. Exemplary bacteriophage tail proteins according to the present invention with a Strep-tag are the SEQ ID NO:8 (N-Strep Miro1p12), SEQ ID NO:10 (N-Strep Miro2p12), SEQ ID NO:12 (N-Strep Effe04p12) and SEQ ID NO:14 (N-Strep Aehl p12) as well as the encoding DNA sequences SEQ ID NO:7 (N-Strep Miro1p12), SEQ ID NO:9 (N-Strep Miro2p12), SEQ ID NO:11 (N-Strep Effe04p12) and SEQ ID NO:13 (N-Strep Aehl p12). The present invention further relates to fragments of bacteriophage tail proteins and the DNA sequences encoding the fragments of the bacteriophage tail proteins according to the present invention. Because the N-terminus of this phage proteins is often responsible for binding to phages and the C-terminus for binding to bacterial receptor, as shown for phage T4 (Makhov AM, et al., Virology. 1993 May;194(1):117-127), in particular C-terminal fragment are preferred. Derivates or fragments are able to be gained e.g. by limited proteolysis (van Raaij MJ, et al., Biol Chem. 2001 Jul;382(7):1049-1055) or randomly genetically produced. Fragments are preferred, which comprises the binding region to the bacterial receptor.

The bacteriophage tail proteins according to the present invention are able to be used for following described methods for removing and for detecting endotoxin. The bacteriophage tail proteins according to the present invention bind endotoxin and are substantially independently of the concentration of bivalent positive ions e.g. Ca^{2+} and/or Mg^{2+} . So the endotoxins may be present in solutions or samples, which contain or which not contain bivalent positive ions. Further substantially independent means, that the solution or sample has no free or bound bivalent positive ions. On the one hand the solution or sample may be totally free of bivalent positive ions. On the other hand it is possible, that the bivalent positive ions are present in the solution or sample bound to substances, which bind bivalent positive ions e.g. EDTA, HEDTA, EGTA, citrate and similar.

There exist two groups of bacteriophage tail proteins which may be differed in whose

binding because of the dependence of bivalent positive ions such as Calcium. P12 bacteriophage tail proteins e.g. of the myoviridae phages as T4, T2, K3, Ox2, RB32-33, AR1, PP01 or RB69 require calcium for binding endotoxin, while the bacteriophage tail protein according to the present invention of the phages Miro1, Miro2 und Effe04 as well
5 as the structural similar proteins to said bacteriophage tail proteins e.g. RB43p12, RB49p12, 44RR2p12, PHG31p12, AehIp12 and KVP40p12 are also able to bind endotoxin without calcium or other bivalent positive ions.

The binding mechanism of calcium-independent bacteriophage tail protein Miro2p12 to
10 endotoxin differs from that of the calcium-dependent bacteriophage tail protein T4p12. T4p12, a calcium dependent bacteriophage tail protein, requires the heptose-Kdo region in the inner core region of endotoxins for binding endotoxin. In contrast, Miro2p12, a calcium independent bacteriophage tail protein, does not requires the heptoses of the inner core region for binding, in fact, Miro2p12 is able to bind also mutants in the core region,
15 which only have the 2-keto-3-deoxyoctonic acid (Kdo), see table 1. The described experiments in figure 5 and figure 6 document these as well, in which Miro2p12 and T4p12 bind endotoxin at the same time and therefore at different binding sides.

The present invention further relates to a method for removing endotoxins from a sample,
20 comprising the steps:

- a. incubating or contacting bacteriophage tail proteins to a sample unspecifically or directed immobilized to a solid carrier, wherein the bacteriophage tail proteins are able to bind endotoxin independently of bivalent positive ion concentrations, and subsequently
- 25 b. separating the endotoxin-bacteriophage tail proteins complex from the sample.

For said depleting method according the present invention, the bacteriophage tail proteins according to the present invention are coupled to solid carrier. The solid carrier may be
30 filling material for chromatography columns (e.g. sepharose materials), cellulose, filtration media, glass particles, magnetic particles, centrifugations materials or sedimentation materials (e.g. agarose particles).

Important hereby is a functional coupling, i.e. bacteriophage tail proteins have despite of the binding to the carrier material accessible structure for endotoxin. The coupling of the bacteriophage tail proteins may be unspecific or however preferably directed, by e.g. a selective biotinylation or coupled by a spacer or a linker.

Besides it is possible that the bacteriophage tail proteins according to the present invention are linked with low molecular substances e.g. biotin to bind to polypeptides e.g. Streptavidin, which are immobilized to a carrier on their parts, by this low molecular substances. Further, instead of biotin it is possible to use said Strep-tag (Skerra, A. & Schmidt, T. G. M., Biomolecular Engineering, 16 (1999), 79-86), which is a short amino acid sequence and binds to Streptavidin. Further it is possible to use the His-tag, which is able to bind to a carrier material by bivalent ions (zinc or nickel) to chelator material as nickel nitrilotriacetate (Ni-NTA, Qiagen, GmbH, Hilden; tolerate no EDTA) or nickel sepharose (General Electric Healthcare/Bio-Sciences/Amersham Biosciences Europe GmbH, Freiburg; tolerate low EDTA concentrations) or a specific antibody for it (Qiagen GmbH, Hilden). The Strep-tag as well as the His-tag is preferably bound by DNA recombination technology to the bacteriophage protein. This coupling may occur directed, e.g. to N-terminus or C-terminus or undirected. The directed coupling occurs by a suitable reactive, usually for phage proteins a not often surface exposed amino acid as cystein, which is inserted at a suitable point. Because bacteriophage tail proteins are synthesized in the cytoplasm, it is not reckon with disulfide bonds. Preferable it is possible to couple direct by other amino acids or by a spacer or cross linker such as cystein (mono functional or bi functional).

During the cystein coupling all bi functional cross linker are possible with NH reactive or SH reactive groups, with or without spacer, e.g. 11-maleimidoundecanoic acid sulfo-NHS or Succinimidyl-4-[N-maleimidomethyl]-cyclohexane-1-carboxy-[6-amido]caproate. Unless there are no spacers, 8-12 C-atom spacers with a terminal NH-group are able to be inserted. Preferable the cystein coupling occurs by a specific biotinylation of the cystein by e.g. EZ-link PEO-Maleimide activated biotin (Pierce).

Further it is possible that the coupling occurs by known coupling reactions to protein residues, e.g. carboxyl, amino, hydroxyl or sulfhydryl residues.

5 The concentration of free not bound bivalent positive ions is preferable less than approximately 0.1 μ M.

The separation of endotoxins can be carried out in a chromatographic method or in the so called batch method. During the batch method the sample charged with endotoxin is mixed and incubated with carrier material, to which the bacteriophage tail proteins
10 according to this invention are coupled covalently.

The separation is carried out after the incubation of the sample with the corresponding with bacteriophage tail protein coupled carrier material for approximately 5-60 min or approximately 30-180 min or over night if required. The duration of the incubation has to
15 be adjusted to the respective sample and may vary between 1 min and 24h. Therefore the sample e.g. is eluted or filtrated from the chromatography column or the corresponding particles are centrifuged or sedimented and magnetically separated by application of a magnetic field, respectively. In particular the separation described in this batch method, i.e. with preincubating the sample and with the corresponding bacteriophage tail protein
20 coupled carrier material, makes sense at very low endotoxin concentrations.

The depletion of endotoxins by chromatography column may also be carried out in pure flow-thorough methods. Therefore the carrier material charged with the bacteriophage tail protein according to the present invention is cast to a chromatography column. For this the
25 sample may be applied onto the column, which comprises the bacteriophage tail proteins coupled to the carrier material. The sample charged with endotoxins is applied onto this column and flows through it, wherein the endotoxin binds to the bacteriophage tail proteins and remains on the column. Ideally the sample itself shows no interactions with the chromatography material and is located in the flow-through. The flow rate is
30 dependent of the volume and the geometry of the column. Further the flow rate is dependent of the volume and the endotoxin content of the sample to achieve also at low endotoxin concentration an efficient depletion by a contact time between column and

endotoxin as long as possible. Thereby the contact time is the time, which the sample needs from the application onto the column until the outflow. The endotoxin bound onto the column may be removed from the column by washing with suitable buffers, so that it is possible to use the column several times.

5

The used bacteriophage tail proteins for said method of removing endotoxins according to this invention may be all preceding described bacteriophage tail proteins according to the present invention and derivatives and fragments thereof, in particular according to SEQ ID NO:2, 4, 6, 8, 10, 12, 14.

10

A further aspect of the present invention regards methods for detecting endotoxin. The endotoxin detection is carried out either by a direct detection of the bacteriophage tail protein bound to endotoxin or indirect by the detection of excessive, not bound bacteriophage tail protein in the supernatant or the flow-through.

15

The direct endotoxin detection comprised the following steps:

- a. contacting the endotoxin containing sample to a surface, and subsequently optionally
- b. removing the sample from the surface, subsequently
- 20 c. incubating of bacteriophage tail proteins with the endotoxins immobilized to the surface, wherein the bacteriophage tail proteins are able to bind endotoxin independently of the bivalent positive ion concentration, and subsequently optionally
- d. removing the unbound bacteriophage tail proteins, and subsequently
- 25 e. detecting of the bacteriophage tail proteins bound to endotoxin

In the first step the binding of endotoxin occurs to the surface. The surface may be coated with an endotoxin binding ligand by means of adsorption or covalent coupling. As surfaces different synthetics are preferred e.g. polystyrene, polypropylene, polyethylene, polycarbonate, PMMA, cellulose e.g. cellulose acetate, nitrocellulose, glass, silicon or
30 agarose. The covalent immobilization of endotoxin binding ligands, e.g. polymyxin B, histamine, histidine, poly-L-lysine, DEAE, polyethylenimin, deoxycholic acid, poly γ -

amino methyl-L-glutamine, polyvinyl alcohol, poly-N,N-dimethylaminopropylacrylamide, dextran, chitosan or calcium independent bacteriophage tail proteins is carried out by known coupling reactions. Alternatively it is possible to bind these ligands by means of biotin-Streptavidin coupling to the surface.

5

The calcium independent bacteriophage tail proteins can be used either for binding endotoxins to the surface or for detecting bound endotoxins. Because endotoxins are normally not monomer but form aggregates in the solution (Mueller M, et al., J Biol Chem. 2004 Jun 18; 279(25):26307-26313) it is also possible to achieve the binding of
10 endotoxins by means of a calcium independent phage tail protein and to use the same calcium independent phage tail protein for detecting endotoxins.

Likewise it is possible to use a combination of Ca dependent and Ca independent bacteriophage tail proteins, wherein one of these acts for immobilization of endotoxin to
15 the surface and the second for the detection of bound endotoxin.

Therefore a further method for detecting endotoxin comprises the following steps:

- a. contacting the endotoxin containing sample to a surface immobilized with first bacteriophage tail proteins, said bacteriophage tail proteins are able to
20 bind endotoxin independently of the bivalent positive ion concentration, and subsequently optionally
- b. removing the sample from the surface, subsequently
- c. incubating of second bacteriophage tail proteins to the endotoxins bound to the first bacteriophage tail proteins, wherein the second bacteriophage tail
25 proteins bind endotoxins only in the presence of bivalent positive ions, and subsequently optionally
- d. removing the unbound second bacteriophage tail proteins and following detection of the second bacteriophage tail proteins bound to endotoxin.

30 The method can also be carried out as the first bacteriophage tail protein is a Ca dependent and the second bacteriophage tail protein is a Ca independent bacteriophage tail protein.

The bacteriophage tail proteins, which should be applied for said detection according to the present invention, have only to have a marker, by whom the detection of the protein is subsequently carried out.

- 5 The endotoxine detection in or from a sample is carried out by the binding of bacteriophage tail proteins to endotoxin. These binding can be detected e.g. by direct measurement via spectroscopic methods, e.g. by means of fluorescence emission, fluorescence polarization, absorption or circular dichroism. Furthermore it is possible to make the binding visible by electric signals, e.g. by a capacity measurement. For
- 10 fluorimetric detection the bacteriophage tail proteins are substituted with fluorescence markers, e.g. fluorescein, Alexa448 or similar. Alternatively, the detection is carried out by a similar method to ELISA, wherein firstly specific antibodies bind to the bacteriophage tail proteins. The detection of these antibodies is carried out by means of enzymes, which are fused either directly with the antibody or with a so-called second
- 15 antibody, which bind to the first antibody. As enzymes alkaline phosphatase or the horseradish peroxidase are considered particularly, but also others. Said enzymatic marker proteins can also be coupled directly to the bacteriophage tail protein. This can be carried out either by the production of fusion proteins or by chemical coupling of both proteins. Alternatively, it is possible to mark the bacteriophage tail proteins with biotin, which may
- 20 be detected by enzymes coupled to Streptavidin, as alkaline phosphatase or horseradish peroxidase.

A further method for the direct detection of endotoxin comprises the following steps:

- 25 a. contacting the endotoxin containing sample to a surface immobilized with first bacteriophage tail proteins, said bacteriophage tail proteins are able to bind endotoxin independently of bivalent positive ion concentration, and subsequently optionally
- b. removing the sample from the surface, subsequently
- c. detecting the endotoxins bound in step a)

30

The detection of endotoxin, which is bound to a Ca independent bacteriophage tail protein, may be carried out by means of an endotoxin specific ELISA or by chemical or enzymatic detection reactions of endotoxins or separated endotoxin components.

The indirect detection comprises the following steps:

- a. contacting the endotoxin containing sample to a surface, and subsequently optionally
- 5 b. removing the sample from the surface, subsequently
- c. incubating of bacteriophage tail proteins with the endotoxins immobilized to the surface, wherein the bacteriophage tail proteins are able to bind endotoxin independently of the bivalent positive ion concentration, and subsequently optionally
- 10 d. removing the unbound bacteriophage tail proteins, and subsequently
- e. detecting of the unbound bacteriophage tail proteins obtained in step d.

If endotoxins are bound to calcium independent bacteriophage tail proteins immobilized to said surface, the method comprises the following steps:

- 15 a. contacting the endotoxin containing sample to a surface immobilized with first bacteriophage tail proteins, said bacteriophage tail proteins are able to bind endotoxin independently of the bivalent positive ion concentration, and subsequently optionally
- b. removing the sample from the surface, subsequently
- 20 c. incubating of second bacteriophage tail proteins to the endotoxins bound to the first bacteriophage tail proteins, wherein the second bacteriophage tail proteins bind endotoxins only in the presence of bivalent positive ions, and subsequently optionally
- d. removing the unbound second bacteriophage tail protein, and subsequently
- 25 e. detecting of the unbound second bacteriophage tail protein obtained in step d.

The binding of the endotoxins occurs either by calcium independent bacteriophage tail proteins, which are immobilized to said surface as described above, or by other endotoxin binding surfaces. The detection of bound endotoxins is carried out by calcium independent or calcium dependent bacteriophage tail proteins, which are added after the binding of the
30 endotoxins to the surface, which have an additional marker, by which they are detected.

These are given in known concentrations to the surface with said endotoxins, are incubated and afterwards the unbound labeled bacteriophage tail proteins are removed or washed out again. By the decrease of the labeled bacteriophage tail proteins in the supernatant or flow-trough the endotoxin amount, which has bound to the surface, is
5 detected.

Furthermore, it is possible to detect endotoxin by a competitive test, in which the labeled endotoxins or labeled endotoxin constituents compete against the endotoxin contained in the sample for binding sides of the calcium independent phage proteins. The endotoxin
10 detection is carried out in this test also indirect by the detection of the calcium independent bacteriophage tail proteins bound to labeled endotoxin or by the labeled endotoxins, which did not bound to the calcium independent bacteriophage tail protein because of the competitive inhibition.

15 The competitive detection comprises the following steps:

- a. mixing the sample with endotoxins, which are coupled to a marker, subsequently
- b. applying the mixture of step a) to a surface with immobilized bacteriophage tail proteins, wherein the bacteriophage tail proteins are able to bind endotoxin independently of the concentration of bivalent positive ions, subsequently
- 20 c. removing the mixture from the surface, subsequently
- d. washing the surface, and subsequently
- e. detecting the labeled endotoxins on the surface and/or the free labeled endotoxin of the pooled samples after step c) and d).

25 The required endotoxins for this detection are gained by known methods for purification of endotoxins (Galanos C., et al., (1969), Eur. J. Biochem. 9, 245-249; Westphal O., Jann K. (1965) In R.L. Whistler (ed.) Methods in carbohydrate chemistry, vol.5, 83-91) and are supplied with markers. For labelling the same marker are used as for the bacteriophage tail protein according to the present invention as fluorescence marker, biotin, digoxigenin,
30 antibodies, enzymatic markers or other markers and with this the corresponding detection methods. Only the coupling, which occurs for endotoxins by the sugar residues, is differently and occurs according to known methods for labeling sugars (Toelstra A. et al. (1997) J. Leukoc. Biol. 61, 170-178; Triantafilou K. et al. (2000), Cytometry 41, 316-

320). The quantification is carried out by a concentration series with a standard endotoxin.

The calcium independent bacteriophage tail protein used in the detections according the present invention may be the bacteriophage tail proteins according to the present invention
5 described above, which also can be used for removing endotoxin.

The following examples are provided merely by way of explanation and in no sense restrict the scope of invention. If not else declared, molecular biologic standard methods were used, e.g. described by Sambrook et al., 1989, Molecular cloning: A Laboratory
10 Manual 2. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Example 1: Effect of calcium on the endotoxin binding of T4p12, N-Strep-Miro2p12 and N-Strep-Aeh1p12.

The short tail fibre proteins T4p12, N-Strep-Miro2p12 and N-Strep-Aeh1p12 were
15 immobilized to NHS activated sepharose 4 Fast Flow (Amersham Biosciences) according to the instruction of the manufacturer and afterwards the binding of endotoxin to said sepharoses was examined. For that purpose columns were cast, a solution with endotoxin was applied onto said columns and the flow-through was collected. The endotoxin content in the application und in the flow-through was determined via LAL-test (kinetic
20 chromogenic LAL-test, Cambrex). The columns had volumes of 1 ml for T4p12 and N-Strep-Miro2p12 and 0.2 ml for N-Strep Aeh1p12. Each 1 ml of a BSA solution (1 mg/ml) was applied onto T4p12 and N-Strep-Miro2p12 columns and 0.2 ml of a buffer solution to N-Strep-Aeh1p12 column. The applied solutions were all studded with endotoxin of E.coli O55:B5 (approximately 1000 EU/ml). To see the effect of calcium to the endotoxin
25 removal and therefore the endotoxin binding, the experiment was carried out with calcium containing buffer and calcium free buffer. The calcium containing buffer consisted of 20 mM Hepes, 150 mM NaCl, 0.1 mM CaCl_2 , pH 7.5 and the calcium free buffer of 20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7.5. The concentration of free calcium ions was 0.1 mM in the calcium containing buffer and under 1 μM in the calcium free buffer with
30 the chelator EDTA (Bers D. et al., in Methods in Cell Biology 40, A practical guide to the study of Ca^{2+} in living cells, chapter 1: A practical guide to the preparation of Ca^{2+} Buffers, Academic Press, 1/94).

As shown in Figure 1, it was only possible to remove endotoxin from the solution in the calcium containing buffer by T4p12 (99.9 % endotoxin removal), while endotoxin was removed from the solution next to nothing in the calcium free buffer (3.2 % endotoxin removal). The endotoxin removal was independent of the calcium concentration in the buffer by N-Strep-Miro2p12 sepharose. It was possible to remove over 99% of endotoxin as well with the calcium containing buffer (99.5% endotoxin removal) as with the calcium free buffer (99.9 % endotoxin removal). It was also not possible to find an effect of the calcium concentration for N-Strep-Aehp12 sepharose. 88.5% of endotoxin was removed from the solution in the calcium containing buffer and 84.3 % in the calcium free buffer.

Example 2: 1. Construction of Miro1, Miro2 and Effe04 with N-terminal Strep-tag: Via PCR the nucleotide sequence for the Strep-tag was added to the 5' end of the Miro2 gene (US Patent 5,506,121). Therefore a primer for the 5' end of the Miro2 gene was constructed (5'-GAA GGA ACT AGT CAT ATG GCT AGC *TGG AGC CAC CCG CAG TTC GAA AAA GGC GCC GCC CAG AAT AAC TAT AAT CAC*-3'; SEQ ID NO:15), which comprises the nucleotide sequence of the Strep-tag at its 5' end (cursive in the sequence) and a restriction recognition side (*NdeI*, underlined in the sequence) in that way, that the gene can be inserted into the right reading frame of the expression plasmid. A primer for the 3' end of the Miro2p12 gene (5'- CG GGA TCC TCC TTA CGG TCT ATT TGT ACA-3'; SEQ ID NO:16) was constructed, which adds a *BamHI* restriction recognition side behind the Miro2p12 gene (underlined in the sequence). The PCR was carried out with 35 cycles (15s 94°C, 15s 51°C, 1 min 74°C). The PCR preparation was restricted with *NdeI* and *BamHI* and the fragment of interest was inserted into the *NdeI* und *BamHI* site of the expressions plasmid pET21a after size fractionation by an agarose gel and elution from the gel. The sequence of the Miro2p12 gene was verified by DNA sequencing. Afterwards the plasmid pNS-Miro2 was transformed into the expression strain BL21 (DE3).

The cloning of Miro1p12 was carried out analog to the described cloning of Miro2p12 above. The same primer and restriction enzymes were used.

The construction and cloning of N-Strep-Effe04p12 was carried out analog to the procedure for N-Strep-Miro2p12 as described above. A primer was used for the 5' end of

the N-Strep-Effe04p12 gene with the sequence 5'-GAA GGA ACT AGT GCT AGC GCT AGC *TGG AGC CAC CCG CAG TTC GAA AAA* GGC GCC AGT AAC AAT ACA ATC AAC CAC G-3' (SEQ ID NO:17), which contains a restriction site for NdeI (underlined in the sequence) and a nucleotide sequence for Strep-tag (cursive). For the 3' end a primer was used with the sequence 5'-CG GGA TCC CCT CTG TTA TAA TAC GCG-3' (SEQ ID NO:18), which comprises a restriction recognition site for BamHI (underlined in the sequence). The PCR preparation was restricted with NdeI and BamHI, inserted into the expression plasmid pET21a and transformed into the expression strain BL21(DE3).

10 Example 3. Purification of N-Strep-Miro2 protein: The E.coli strain BL21(DE3) was raised with the plasmid pNS-Miro2 in a 2 l agitation culture (LB-medium with ampicillin, 100 µg/ml) until a OD600 of 0.5-0.7 at 37°C and the expression of the N-Strep-Miro2 protein was induced by the addition of 1 mM IPTG (isopropyl-β-thiogalactopyranoside). After incubation at 37°C for 4h the cells were harvested. Harvest cells of 10 l culture were
15 sustained into 50 ml 10 mM sodium phosphate, pH 8.0, 2 mM MgCl₂, 150 mM NaCl, disrupted by a French-Press treatment (20.000 psi) for three times and afterwards centrifuged for 30 min at 15.000 rpm (SS34). After washing for two times in the same buffer, the N-Strep-Miro2 protein was extracted from the pellet by stirring for 30 min in 10 mM TrisHCl pH 8.0, 150 mM NaCl, 1 M urea, the preparation was centrifuged for 30
20 min at 15.000 rpm (SS34) and the released N-Strep-Miro2 was embedded in the supernatant at 4°C. The extraction was repeated twice. The pooled supernatants were applied onto a streptactin affinity column (15 ml) equilibrated with buffer „W“ (100 mM TrisHCl pH 8, 1 mM EDTA, 150 mM NaCl), (IBA GmbH, Göttingen, Germany). After washing with 5 column volumes of buffer “W” it was eluted with 3 column volume buffer
25 „W“ with 2.5 mM desthiobiotin in buffer „W“. After dialyse against buffer “W” for several times and concentration, the concentration and purity of N-Strep-Miro2 was examined by SDS-PAGE and UV spectroscopy. Approximately 100 mg N-Strep-Miro2 were so purified from 10 liter culture.

30 Example 4: Detection of the binding of different long endotoxin variations to N-Strep-Miro2p12 via surface plasmon resonance spectroscopy. This experiment conduces for the purpose to get information of the structure in the core region of endotoxin recognized by

Miro2p12. N-Strep-Mirop12 was covalent coupled to the surface of a cell on a CM-5 Chip by company Biacore. Therefore at first the surface was activated with EDC/NHS, afterwards N-Strep-Miro2p12 was bound by primary amino residues and finally unused coupling groups were saturated with ethanol amine (see Biacore handbook). Endotoxin was isolated from *E. coli* strains, which have unequal long core sugars (see Figure 9, Lit.: Boman H.G., Jonsson S., Monner D., Normark S., Bloom G.D., Cell-Surface alterations in *Escherichia coli* K-12 with chromosomal mutations changing ampicillin resistance. Ann. N.Y. Acad. Sci. 1971; 182: 342-357; Prehm P. Stirm S., Jann B., Jann K., Bomann H.G., Cell-wall lipopolysaccharides of ampicillin-resistant mutants of *Escherichia coli* K-12. Eur. J. Biochem. 1976; 66(2): 369-377; Eriksson-Grennberg K.R., Nordstrom K., Englund P., Resistance of *Escherichia coli* to penicillins. IX. Genetics and physiology of class II ampicillin-resistant mutants that are galactose negative or sensitive to bacteriophage C21, or both. J. Bacteriol. 1971; 108(3): 1210-1223; Boman H.G., Monner D.A., Characterization of lipopolysaccharides from *Escherichia coli* K-12 mutants. J. Bacteriol. 1975; 121(2): 355-464). Therefore the bacteria were raised in LB-medium at 37°C over night, harvested by centrifugation, washed with PBS and afterwards the pellet was sustained into 100 mM Tris, 50 mM EDTA, pH 8 and incubated for 30 min at room temperature. In this buffer a part of the endotoxin is solved and can be separated from the cells. Afterwards the solved endotoxin was precipitated with four times volume of acetone and dried. For detecting endotoxin was sustained into running buffer (running buffer: 20 mM Hepes, 150 mM NaCl, 0.005% Tween 20, pH 7.5) and rinsed over the surface loaded with N-Strep-Miro2p12. A second unloaded cell on the Biacore Chip was used as a control. The endotoxin solution was applied onto both cells during the measurement and the resulting signal was calculated from the signal differences between cell1 and cell2. The increase of the signal in the resulting curve was interpreted as the binding of endotoxin to the N-Strep-Miro2p12. As shown in table 1, it was also possible to detect a binding with endotoxin from the *E.coli* strain D21f2. This stain comprises only the Kdo-Core sugars und therefore the shortest endotoxin form, which is absolute necessary for the survival of the cells. It was not possible to detect a binding of said bacteria with T4p12. Thereby the addition R means, that these bacteria belong to the so-called rough type whose endotoxin have no O-antigen and the small letters a to e mark the decreasing length of core sugars in the endotoxin. The corresponding core sugars are drafted in figure 9.

Table 1		
bacteria strain	binding to T4p12	binding to N-Strep-Miro2p12
<i>E. coli</i> D21, Ra	+	+
<i>E. coli</i> D21e7, Rb	+	n.d.
<i>E. coli</i> D21e8, Rc	+	n.d.
<i>E. coli</i> D21f1, Rd	+	+
<i>E. coli</i> D21f2, Re	-	+
		n.d. = not determined

Example 5: Endotoxin removal by the bacteriophage tail protein Miro2.

- 5 5 ml NHS activated sepharose 4 Fast Flow (Amersham Biosciences) was centrifuged, the isopropanole supernatant was removed and mixed with 870 ml citrate buffer (25 mM citrate, 2 mM EDTA, pH 7.0). Afterwards 217 ml bacteriophage tail protein Miro2 (0.46 mg/ml in 50 mM formiate, pH 3.5) were added und shacked for 2 hour at 37°C for coupling the bacteriophage tail protein to the sepharose. The supernatant was removed, the
- 10 sepharose was washed with 10 mM sodium phosphate, pH 10 for three times and 1 ml aliquots of supernatant and wash fractions were dialyzed against 10 mM sodium phosphate, pH 10. The concentration of bacteriophage tail protein in these aliquots was determined via absorption measurement at 280 nm and the amount of bacteriophage tail protein bound to the sepharose was calculated. 12.2 mg bacteriophage tail protein were
- 15 bound per 1 ml sepharose.

Columns were cast with a volume of 1.5 ml. Moreover columns were cast with the same volume of T4p12 material and polymyxin B sepharose. Each one of these columns was equilibrated with running buffer (20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7.5)

20 and afterwards the endotoxin removal from a BSA solution (1.2 mg/ml) charged with lipopolysaccharide of *E. coli* O55:B5 (approximately 1000 EU/ml) was determined. Therefore before the application of the sample, at first 1 ml running buffer was collected

from each column (Miro2p12, T4p12, Polymyxin B) and afterwards each 1 ml BSA was applied and washed with running buffer. The flow-through was fractionated and the fractions were determined of protein (BSA) and endotoxin. The results are shown in figure 2. No endotoxin was able to be removed from the solution by T4p12, which requires free calcium for binding endotoxin. It was possible to reduce the endotoxin content about 96% by polymyxin B but only 67 % of the applied BSA was recovered. It was possible to remove more than 99% of endotoxin by Miro2p12 and the recovery rate for BSA was 92% and was therefore significantly higher than for polymyxin B.

Similar experiments were carried out with PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and citrate (20 mM citrate, 150 mM NaCl, pH 7.0) as running buffers. Also in these buffers the endotoxin removal was strongly decreased by T4p12, because calcium was either precipitated (phosphate buffer) or complexed (citrate). The results are shown in the figures 3 and 4 and show expectedly a low (PBS) and no endotoxin removal (citrate), respectively, by T4p12. It was possible to remove each time more than 96% of endotoxin by polymyxin B, but the BSA recovery was only between 60-80%. It was possible to remove more than 99% of endotoxin by Miro2p12 and the BSA recovery was higher than 90%. Moreover the suitability of N-Strep-Miro2p12 was determined for the endotoxin removal from serum. Therefore human serum was studied with lipopolysaccharide of *E. coli* O55:B5 and applied onto N-Strep-Miro2p12 sepharose columns (see figure 7). It was possible to remove approximately 90% of the applied endotoxin from human serum.

Example 6: Detection of LPS by the binding of T4p12 to immobilized LPS.

Bacteriophage tail protein Miro2p12 was immobilized to NHS-Sepharose (Amersham Pharmacia) (3.4 mg protein per 1 ml sepharose) and out of this 4 column were cast with a volume of 0.5 ml each. The columns were washed with each 3 ml sodium phosphate buffer (20 mM sodium phosphate, pH 12.0) and with each 3 ml regeneration buffer (20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7.5). Afterwards each 1 ml LPS of *E. coli* O55:B5 was applied onto two of these columns (0.1 mg/ml in Hepes buffer, 10⁶ EU/ml). The two other columns were rinsed with each 1 ml regeneration buffer. Following all

columns were washed with each 3 ml equilibration buffer (20 mM Hepes, 150 mM NaCl, 0.1 mM CaCl₂, pH 7.5) and afterwards 1 ml of this buffer was applied again and collected as fraction 1. Following 0.5 ml of a solution with the bacteriophage tail proteins T4p12 (0.844 mg/ml in 20 mM Hepes, 150 mM NaCl, 0.1 mM CaCl₂) was applied onto the columns and washed with 2.5 ml equilibration buffer and 2 ml regeneration buffer. The flow-through was collected three times in fractions of 1 ml and once of 2 ml and the concentrations of the bacteriophage tail protein T4p12 was determined via absorption measurement at 280 nm in these fractions (figure 5). Most of the bacteriophage tail protein T4p12 was bound onto the columns, which was treated with LPS in advance and was able to be dissolved from these columns by the addition of regeneration buffer. In contrast to the columns not treated with LPS it flows through the columns without delay.

Example 7: 1. Construction of Aeh1 with N-terminal Strep-tag (N-Strep-Aeh1p12): The nucleotide sequence for the Strep-tag (US Patent 5,506,121) was inserted into the 5' end of Aeh1p12-Gens (NCBI Acc.Nr.AAQ17871) by PCR. Therefore a primer was designed for the 5' end of Aeh1 gene (5'-GAA GGA ACT AGT CAT ATG GCT AGC *TGG AGC CAC CCG CAG TTC GAA AAA GGC GCC AGA ACA AAT AAT ATC ACA CAG* 3'; SEQ ID NO:19), which comprises the nucleotide sequence of the Strep-tag at its 5' end (cursive in the sequence) and a restriction recognition site (*NdeI*, underlined in the sequence) in that way, that the gene can be used in the right reading frame in the expression plasmid. For the 3' end of Miro2p12 gene a primer was designed (5'-GAA GGA ACT AGT CAT ATG AGA ACA AAT AAT ATC ACA CAG 3'; SEQ ID NO:20), which inserts a *BamHI* restriction recognition site (underlined in the sequence) behind the Aeh1p12 gene. The PCR was carried out with 35 cycles (15s 94°C, 15s 51°C, 1 min 74°C). The PCR preparation was restricted with *NdeI* and *BamHI* and the fragment of interest was inserted into the *NdeI* und *BamHI* site of the expressions plasmid pET21a after size fractionation by an agarose gel and elution from the gel. The sequence of Aeh1p12 gene was verified by DNA sequencing. Afterwards the plasmid pNS-Aeh1 was transformed into the expression strain HMS174 (DE 3).

2. Purification of N-Strep-Aeh1 protein: The E.coli strain HMS174 (DE 3) was raised

with the plasmid pNS-Aeh1p12 in a 2 l agitation culture (LB-medium with ampicillin 100 µg/ml, kanamycin 25 µg/ml, rifampicin 10 µg/ml) until an OD600 of 0.5-0.7 at 37°C and the expression of N-Strep-Aeh1p12 protein was induced by the addition of 1 mM IPTG (isopropyl-β-thiogalactopyranoside). After incubation at 37°C for 4h the cell were
5 harvested. Harvested cells of 10 l culture were sustained into 50 ml 10 mM sodium phosphate, pH 8.0, 2 mM MgCl₂, 150 mM NaCl, disrupted in a microfluidizer (Microfluidics, M110L) and afterwards centrifuged for 30 min at 15.000 rpm (SS34). After washing for two times in the same buffer the N-Strep-Aeh1p12 protein was extracted from the pellet by stirring for 30 min in 50 mM sodium phosphate pH 12 and the
10 preparation was centrifuged for 30 min at 15.000 rpm (SS34). The extraction was repeated once and the pooled supernatants with the released N-Strep-Aeh1p12 were dialysed against 100 mM Tris, 150 mM NaCl, pH 8.0. Afterwards the protein was further purified by a streptactin affinity column (5 ml, IBA GmbH, Göttingen, Germany). Therefore the streptactin affinity column was equilibrated with buffer „W“ (100 mM TrisHCl pH 8, 1
15 mM EDTA, 150 mM NaCl) and Aeh1p12 was applied. After washing with 5 column volumes of buffer “W” it was eluted with 3 column volume buffer „W“ with 2.5 mM desthiobiotin in buffer „W“. After dialyse against 100 mM borate, 150 mM NaCl, pH 8 for several times, the concentration and purity of N-Strep-Aeh1p12 was examined. So approximately 20 mg N-Strep-Aeh1p12 were purified from 4 liter culture.

20
3. Coupling of Aeh1p12 to NHS activated sepharose. 22 ml N-Strep-Aeh1p12 (0.9 mg/ml in 100 mM borate, 150 mM NaCl, pH 8) were transferred with 200 µl NHS activated sepharose and incubated on a roller for 3h at room temperature. Afterwards the sepharose was centrifuged (15 min, 3000g) and washed with each 20 ml 100 mM Tris, 150 mM
25 NaCl, pH 8 for three times. Following 0.5 ml aliquots of the wash fractions were dialysed against 100 mM Tris, 150 mM NaCl, pH 8 to remove the released NHS and the concentration of N-Strep-Aeh1p12 was determined in these aliquots by absorption measurement at 280nm. The population density was calculated from the output protein amount and the protein amount in the wash fractions. It was possible to couple 2.5 mg N-
30 Strep-Aeh1p12 per 1 ml sepharose.

4. Endotoxin removal by Aeh1p12 sepharose. A column with Aeh1p12 sepharose and a control column were cast, both with each a volume of 200 μ l. The control column consisted of NHS sepharose, which was saturated with ethanol amine. Following both columns were washed with 2 ml 0.5 % sodium deoxycholate, 15 ml pyrogene free water and 2 ml running buffer. Each 200 μ l lipopolysaccharide of *E. coli* O55:B5, solved in the corresponding running buffer, was applied as sample. After the application of sample it was mixed with sepharose and incubated for 30 min at room temperature before the solution runs through the column. At first the columns were washed with 0.6 ml and following also with 1 ml running buffer twice. The flow-through was fractionated and the endotoxin concentration was determined in the application and the fractions via LAL test (kinetic chromogenic LAL-Test, Cambrex). This experiment was carried out with 2 different running buffers to examine the influence of calcium for the endotoxin removal. The running buffers were composed as following: buffer 1: 20 mM Hepes, 150 mM NaCl, 0.1 mM CaCl_2 , pH 7.5. buffer 2: 20 mM Hepes, 150 mM NaCl, 2 mM EDTA, pH 7.5. As shown in figure 8, significantly more endotoxin is removed by the Aeh1p12 sepharose column (88% and 84%) as by the unloaded control column (25% and 39%). Calcium is irrelevant for the binding of endotoxin to Aeh1p12, because the endotoxin removal was in the presence of calcium similar (88%) to a buffer with a calcium chelator (84%).

20 Example 8:

1. Purification of N-Strep-Effe04p12. The *E. coli* strain HMS174 (DE 3) with the plasmid pNS-Effe04p12 was raised in 2 l shaking cultures (LB medium with ampicillin 100 μ g/ml, rifampicin 10 μ g/ml) until a OD600 of 0.5-0.7 at 37°C and the expression of N-Strep-Effe04p12 protein was induced by the addition of 1 mM IPTG (isopropyl- β -thiogalactopyranoside). After incubation at 37°C for 4h the cells were harvested. Harvested cells from 6 l culture were sustained into 50 ml 100 mM Tris, 25 mM EDTA, 100 mM NaCl, pH 8.0, disrupted in a micro fluidizer (Microfluidics, M110L) and afterwards centrifuged for 30 min at 15.000 rpm (SS34). Following the N-Strep-Effe04p12 protein was extracted from the pellet by stirring for 2 h at 37°C in 100 mM Tris, 1.5 M GdnHCl, pH 8.0, and the preparation was centrifuged for 30 min at 13.000 rpm (SS34). The extraction was repeated once. The pooled supernatants with the solved N-Strep-Effe04p12 were dialysed against 100 mM Tris, pH 8.0. Following the protein was

further purified by a streptactin affinity column (5 ml, IBA GmbH, Göttingen, Germany). Therefore the streptactin affinity column was equilibrated with buffer „W“ (100 mM TrisHCl pH 8, 1 mM EDTA, 150 mM NaCl) and N-Strep-Effe04p12 was applied. After washing with 5 column volumes with buffer “W”, it was eluted with 3 column volume buffer „W“ with 2.5 mM desthiobiotin in buffer „W“. After dialyse against 100 mM borate, 150 mM NaCl, pH 8 for several times the concentration and purity of N-Strep-Effe04p12 was examined by SDS-PAGE and UV spectroscopy. So approximately 2 mg N-Strep-Effe04p12 were purified from 6 liter culture.

2. Coupling of N-Strep-Effe04p12 to NHS activated sepharose. 4 ml N-Strep-Effe04p12 (0.2 mg/ml in 100 mM borate, 150 mM NaCl, pH 8) were transferred with 100 µl NHS activated sepharose and incubated on a roller for 3h at room temperature. Afterwards the sepharose was centrifuged (15 min, 3000g) and washed with each 20 ml 100 mM Tris, 150 mM NaCl, pH 8 for three times. Following 0.5 ml aliquots of the wash fractions were dialysed against 100 mM Tris, 150 mM NaCl, pH 8 to remove the released NHS and the concentration of N-Strep-Aehl1p12 was determined in said aliquots by absorption measurement at 280nm. The population density was calculated from the output protein amount and the protein amount in the wash fractions. It was possible to couple 3.6 mg N-Strep-Effe04p12 per 1 ml sepharose.

3. Endotoxin removal by N-Strep-Effe04p12 sepharose. 100 µl N-Strep-Effe04p12-sepharose was applied onto a mini-spin column (Handee Mini-Spin, Pierce) and at first washed with 1 ml endotoxin free running buffer (20 mM Hepes, 150 mM NaCl, 2 mM EDTA) for three times. Therefore the solution was separated from the sepharose by short centrifugation (400g, 5s). Afterwards 100µl endotoxin solution (lipopolysaccharide of *E. coli* O55:B5 in running buffer) was incubated for 30 min with the sepharose before the solution was centrifuged and the sepharose was washed with 200 µl running buffer another two times. The endotoxin amounts were determined as well in the application (F0) as in the fractions after the sepharose by LAL test (kinetic chromogenic LAL test, Cambrex). As shown in figure 9, most of applied endotoxin is removed from the solution (endotoxin removal: 92%). Calcium is irrelevant for the binding of endotoxin to N-Strep-Effe04p12 because EDTA was contained in the running buffer.

Example 9: Endotoxin detection by the binding of Miro2p12 to immobilized LPS.

Bacteriophage tail protein Miro2p12 was immobilized to NHS-Sepharose (Amersham Pharmacia) (5 mg protein per 1 ml sepharose) and out of this 4 column were cast with a volume of 1 ml each. Two of these were charged with LPS of *E.coli* O55:B5 (10^6 EU in 1ml PLS buffer, 10 mM sodium phosphate, 70 mM NaCl, pH 7.4) (+ ET, black bars figure 12), 2 were used as controls (- ET, white bars figure 12). 10 mM sodium phosphate, 80 mM NaCl, pH 7.4 was used as running buffer. Miro2p12 was applied onto all columns (each 600 μ l of a solution with 0.1 mg/ml protein). The amount of applied and eluted Miro2p12, respectively, was determined by absorption at 280 nm. The amount of bacteriophage tail protein Miro2p12 was plotted against the fractions of the chromatography run, as illustrated in figure 12. Fraction 3 (F3) shows the flow-trough of Miro2p12 after the application (F0), so all bacteriophage tail protein, which is not retained by the column, fraction 4 (F4) is a wash fraction. After the washing regeneration buffer RB (10 mM sodium phosphate, 500 mM NaCl, pH 7.4) was added (see arrow direction), which dissolves Miro2p12 bound to endotoxin from the column again. Subsequent the fractions 5 and 6 were collected. Fraction F3 has a volume of 0.6 ml all other fractions have a volume of 1 ml. The application onto the column (F0) with the total amount of Miro2p12 is plotted as control. It can be seen, that the columns, to which endotoxin was immobilized before, Miro2p12 was retained, while only a small amount of Miro2p12 was bound unspecifically to the control columns without endotoxin.

Example 10: Endotoxin detection adsorbed to a PolySorp microtiter plate by the binding of Miro2p12 labeled with biotin.

Production of Miro2p12 labeled with biotin. 2 ml of a Miro2p12 solution with a concentration of 2 mg/ml in a buffer with 50 mM sodium borate, 1.75 M GdmCl, pH 8.0 were transferred with 250 μ l of a 10 mM Sulfo-NHS-LC-LC-biotin solution in water (Pierce) and incubated for 30 min at room temperature. Following the reaction solution is dialysed against buffer with 50 mM sodium borate, 1.75 M GdmCl, pH 8.0. The so obtained Miro2p12-bio was used for the following endotoxin detection, which is illustrated in figure 13. LPS of *E. coli* O55:B5 (3 μ g/ml) was immobilised to PolySorp plates (Nunc) by adsorption (18h at room temperature in PBS-Puffer, 10 mM Na_2HPO_4 ,

1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4). Afterwards the microtiter plates were blocked with casein (0.05 % in PBS, 1.5 h at RT) and washed with PBS buffer once. Control plates were not incubated with endotoxin but only blocked with casein. Each 200 μl Miro2p12 labeled with biotin (Miro2p12-bio) in 50mM Tris, pH 8, 0.05% Casein, 0.05% Tween20 was added in raising concentration (white bars: plates without ET, black bars: plates with ET, protein concentration as specified). After 5 h incubation at room temperature it was washed with each 200 μl PBST (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, 0.05 % Tween, pH 7.4) for three times. The detection of Miro2p12 labeled with biotin and bound to endotoxin was carried out by a biotin specific ELISA. A conjugate of strepavidin with alkaline phosphatase (Amersham Biosciences) is diluted 1:5000 in PBST and each 200 μl of these are incubated with bound Miro2p12-bio for 1.5 h at room temperature. Afterwards they are washed with each 200 μl Tris-T (50 mM Tris, 0.05 % Tween, pH 8.0) for three times. The colorimetric detection is carried out by absorption measurement at 405 nm after the addition of pNPP (para-nitrophenyl phosphate) in a concentration of 0.8 mg/ml as chromogenic substrate. Miro2p12 labeled with biotin binds in a concentration dependent form to the microtiter plates, which were coated with endotoxin beforehand.

Example 11: Detection of FITC labeled endotoxin bound to immobilize Miro2p12.

Miro2p12 (each 200 μl with 5 $\mu\text{g}/\text{ml}$ protein) was adsorbed to a MaxiSorp plate (Nunc) (16 h at RT in PBS, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4). Afterwards the microtiter plates were blocked with casein (0.05 % Casein in PBS, 1.5h at RT, each 200 μl per well) and washed with PBS buffer once. Control plates were not incubated with Miro2p12, but only blocked with casein. Each 100 μl FITC labeled LPS of *E. coli* O55:B5 (Sigma) in PBS was added in raising concentration (white bars: plates without Miro2p12, black bars: plates with Miro2p12). It was washed with each 200 μl PBST (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, 0.05 % Tween, pH 7.4) for three times. The experiment is shown in figure 14. The detection of endotoxin bound to bacteriophage tail protein was carried out in a FITC specific ELISA. Anti-FITC antibodies (0.5 $\mu\text{g}/\text{ml}$, Zymed) are diluted 1:500 in PBST and afterwards each 200 μl were incubated with the FITC labeled endotoxin for 1 h at room temperature. Following it is washed with each 200 μL PBST for three times. As secondary antibody an

anti-rabbit-IgG alkaline phosphatase conjugate is used (1 μ g/ml, Pierce). It is applied in a dilution of 1:5000 and incubated for 1.5 h at room temperature. Afterwards it is washed with each 200 μ l PBST for three times. The quantification is carried out by fluorescence measurement of the reaction products of a fluorescent alkaline phosphatase substrate (methylumbelliferyl phosphate; Sigma) with 0.1 mg/ml methylumbelliferyl phosphate in 50 mM Tris, pH 8.0. Fluorescence labeled endotoxin binds in a concentration dependent form to the microtiter plates, which were coated with Miro2p12 beforehand.

DEMANDE OU BREVET VOLUMINEUX

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CLAIMS:

1. A method for removing endotoxins from a sample, comprising the steps:
 - a) incubating or contacting bacteriophage tail proteins with a sample unspecifically or directedly immobilized to a solid carrier, wherein the bacteriophage tail proteins are able to bind endotoxin independently of the bivalent positive ion concentrations, and subsequently
 - b) separating the endotoxin-bacteriophage tail proteins complex formed from the sample.
2. The method according to claim 1, wherein the steps a) and b) are carried out in a column chromatography flow-through method.
3. The method according to claim 1, wherein the solid carrier is cellulose, filtration media, glass particles, magnetic particles, centrifugation or sedimentation substances or packing material for chromatography columns.
4. The method according to any one of claims 1 to 3, wherein the bacteriophage tail proteins are immobilized to the solid carrier by coupling groups.
5. The method according to any one of claims 1 to 4, wherein the bacteriophage tail proteins are covalent immobilized to the solid carrier by chemical bonds.
6. The method according to any one of claims 1 to 5, wherein the bacteriophage tail protein comprises a Strep-tag or a His-tag.
7. The method according to any one of claims 1 to 6, wherein the bacteriophage tail protein is a short bacteriophage tail protein.
8. The method according to any one of claims 1 to 7, wherein the bacteriophage tail protein derives from the myoviridae family.
9. The method according to any one of claims 1 to 8, wherein the bacteriophage tail

protein is selected from the group consisting of pseudo-T-even, schizo-T-even, and T-even phage.

10. The method according to any one of claims 1 to 9, wherein the bacteriophage tail protein is able to bind the 2-keto-3-deoxyoctonic acid in the core region of the endotoxins.

11. The method according to any one of claims 1 to 10, wherein the bacteriophage tail protein has a sequence selected from SEQ ID NO: 2, 4, 6, 8, 10, 12 and 14.

12. The method according to any one of claims 1 to 11, wherein the concentration of free unbound bivalent positive ions is less than 0.1 μM .

13. The method according to any one of claims 1 to 12, wherein the bivalent positive ions are Ca^{2+} and/or Mg^{2+} .

14. A polypeptide having a sequence according to SEQ ID NO: 2, 4, 6, 8, 10, 12 or 14.

15. A nucleic acid molecule having a sequence according SEQ ID NO: 1, 3, 5, 7, 9, 11 or 13.

16. A method for the detection of endotoxin, comprising the steps:

- a) contacting an endotoxin containing sample with a surface, subsequently
- b) incubating bacteriophage tail proteins with endotoxins immobilized to the surface, wherein the bacteriophage tail proteins are able to bind endotoxin independently of the bivalent positive ion concentration, and subsequently
- c) detecting the bacteriophage tail proteins bound to the endotoxins.

17. The method according to claim 16, further comprising the additional step after step a) and before step b):

- a') removing the sample.

18. The method according to claim 16 or 17, further comprising the additional step after step b) and before step c):

- b') removing the unbound bacteriophage tail proteins.
19. A method for the detection of endotoxin, comprising the steps:
- a) contacting an endotoxin containing sample with a surface, subsequently
 - b) incubating bacteriophage tail proteins with endotoxins immobilized to the surface, wherein the bacteriophage tail proteins are able to bind endotoxin independently of the bivalent positive ion concentration, and subsequently
 - c) removing the unbound bacteriophage tail proteins, and subsequently
 - d) detecting the unbound bacteriophage tail proteins obtained in step c).
20. The method according to claim 19, further comprising the additional step after step a) und before step b)
- a') removing the sample from the surface.
21. The method according to any one of claims 16 to 20, wherein the surface is coated with an endotoxin binding ligand by adsorption or covalent coupling.
22. The method according to claim 21, wherein the endotoxin binding ligand is a bacteriophage tail protein, which is able to bind endotoxin independently of the bivalent positive ion concentration.
23. A method for the detection of endotoxin, comprising the steps:
- a) contacting an endotoxins containing sample with a surface immobilized with first bacteriophage tail proteins, which are able to bind endotoxin independently of the bivalent positive ion concentration, and subsequently
 - b) optionally removing the sample from the surface, subsequently
 - c) incubating second bacteriophage tail proteins with endotoxins bound to the first bacteriophage tail proteins, wherein the second bacteriophage tail proteins bind endotoxins only in the presence of bivalent positive ions, and subsequently
 - d) optionally, removing the unbound second bacteriophage tail proteins, and subsequently
 - e) detecting the second bacteriophage tail proteins bound to the endotoxins.

24. A method for the detection of endotoxin, comprising the steps:
- a) contacting an endotoxins containing sample with a surface immobilized with bacteriophage tail proteins, which are able to bind endotoxin independently of the bivalent positive ion concentration, and subsequently
 - b) optionally, removing the sample from the surface, and subsequently
 - c) detecting the endotoxins bound in step a).
25. The method according to any one of claims 16 to 24, wherein the detection is carried out by spectroscopic methods, ELISA, chemical or enzymatic detection reaction of endotoxins or separated endotoxin components, or by capacity measurement.
26. A method for the detection of endotoxin, comprising the steps:
- a) mixing a sample with endotoxins coupled to a marker, subsequently
 - b) applying the mixture of step a) onto a surface with immobilized bacteriophage tail proteins, wherein the bacteriophage tail proteins are able to bind endotoxin independently of the bivalent positive ion concentration, subsequently
 - c) removing the mixture from the surface, subsequently
 - d) washing the surface, and subsequently
 - e) detecting the labeled endotoxins on the surface and/or the free labeled endotoxins of the pooled samples after step c) and d).
27. The method according to any one of claims 16 to 26, wherein the bacteriophage tail proteins are immobilized to the solid carrier by coupling groups.
28. The method according to any one of claims 16 to 27, wherein the bacteriophage tail proteins are covalently immobilized to the solid carrier by chemical bonds.
29. The method according to any one of claims 16 to 28, wherein the bacteriophage tail protein comprises a Strep-tag or a His-tag.
30. The method according to any one of claims 16 to 29, wherein the bacteriophage tail protein is a short bacteriophage tail protein.

31. The method according to any one of claims 16 to 30, wherein the bacteriophage tail protein derives from the myoviridae family.
32. The method according to any one of claims 16 to 31, wherein the bacteriophage tail protein is selected from the group consisting of pseudo-T-even, schizo-T-even, and T-even phage.
33. The method according to any one of claims 16 to 32, wherein the bacteriophage tail protein is able to bind the 2-keto-3-deoxyoctonic acid in the core region of endotoxins.
34. The method according to any one of claims 16 to 33, wherein the bacteriophage tail protein has a sequence selected from SEQ ID NO: 2, 4, 6, 8, 10, 12 and 14.

Fig. 1

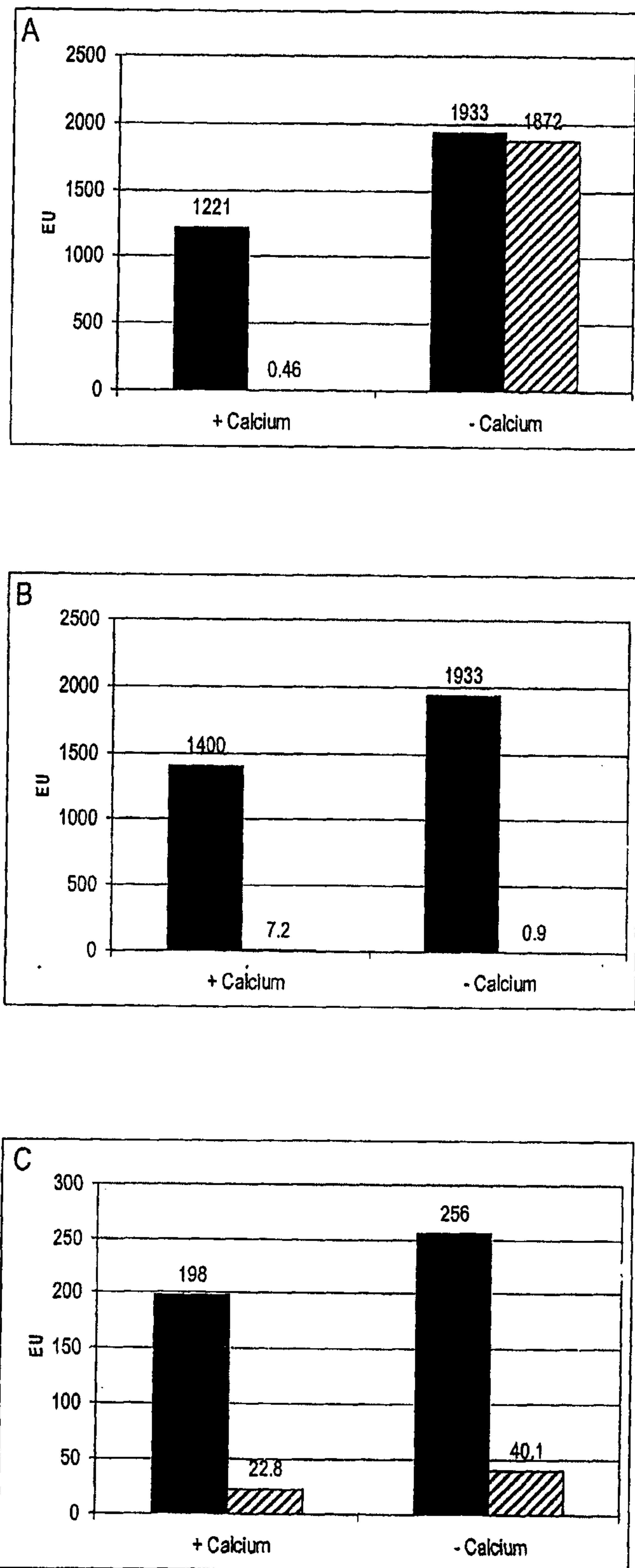


Fig. 2

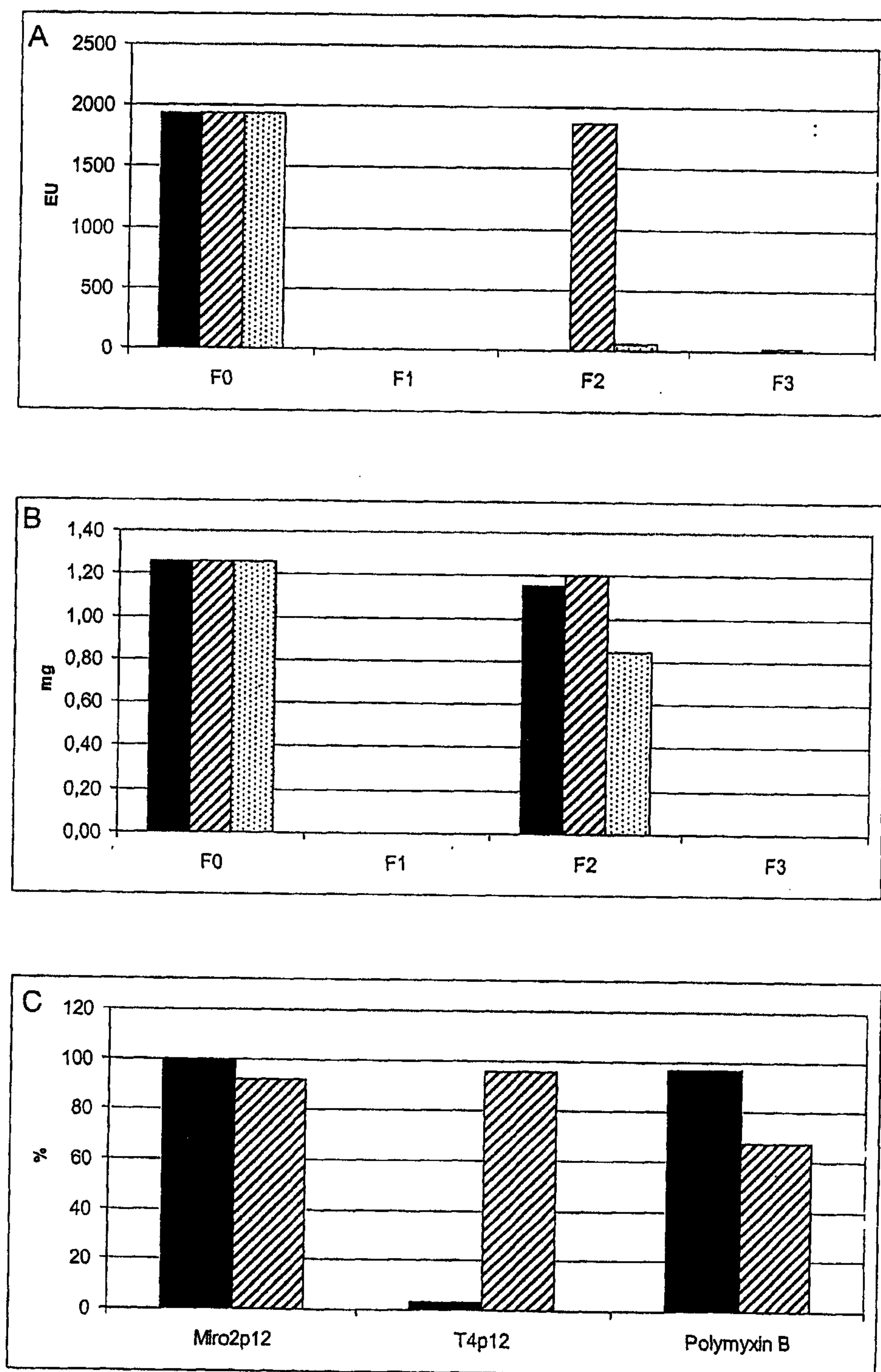


Fig. 3

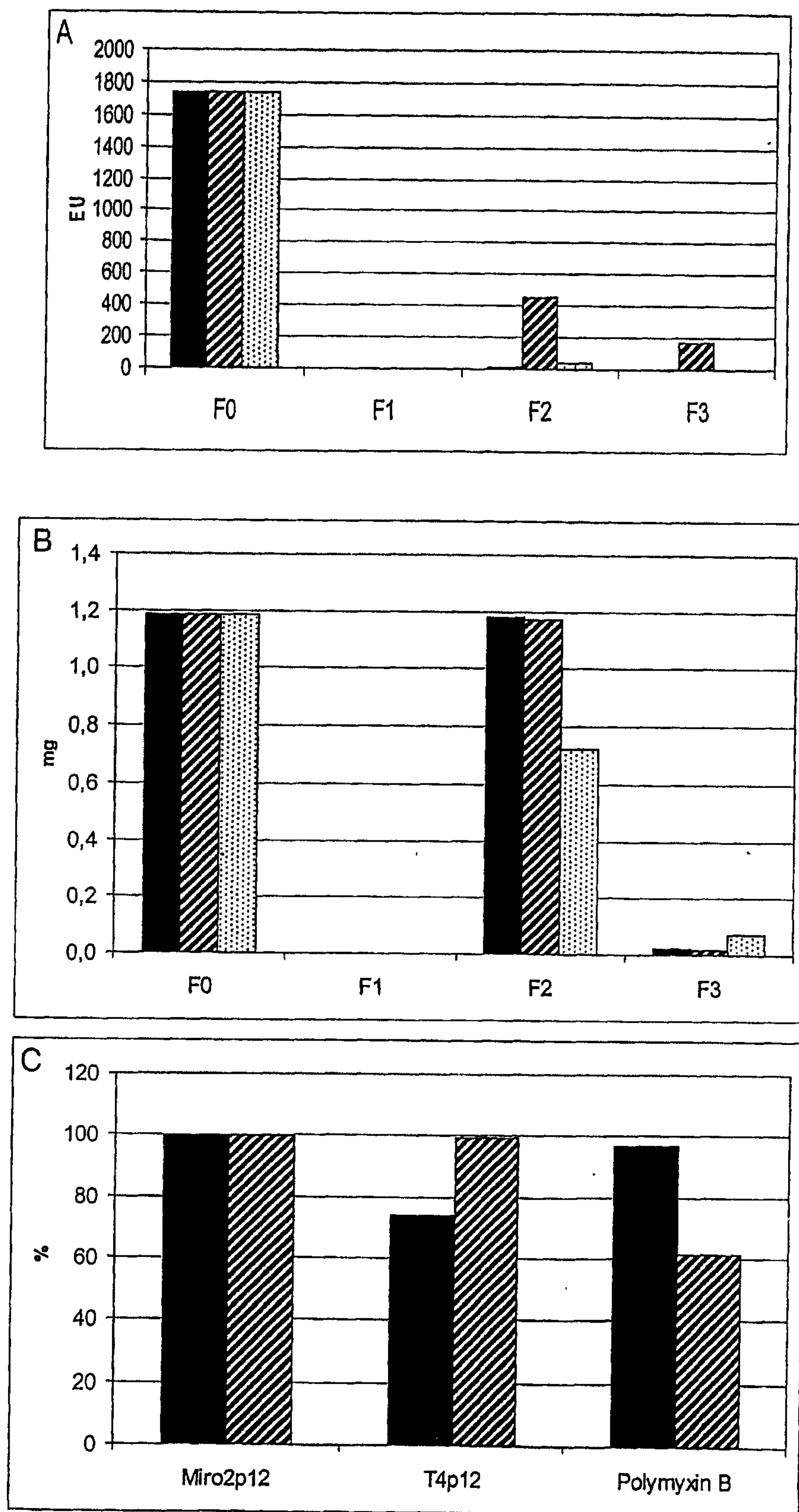


Fig. 4

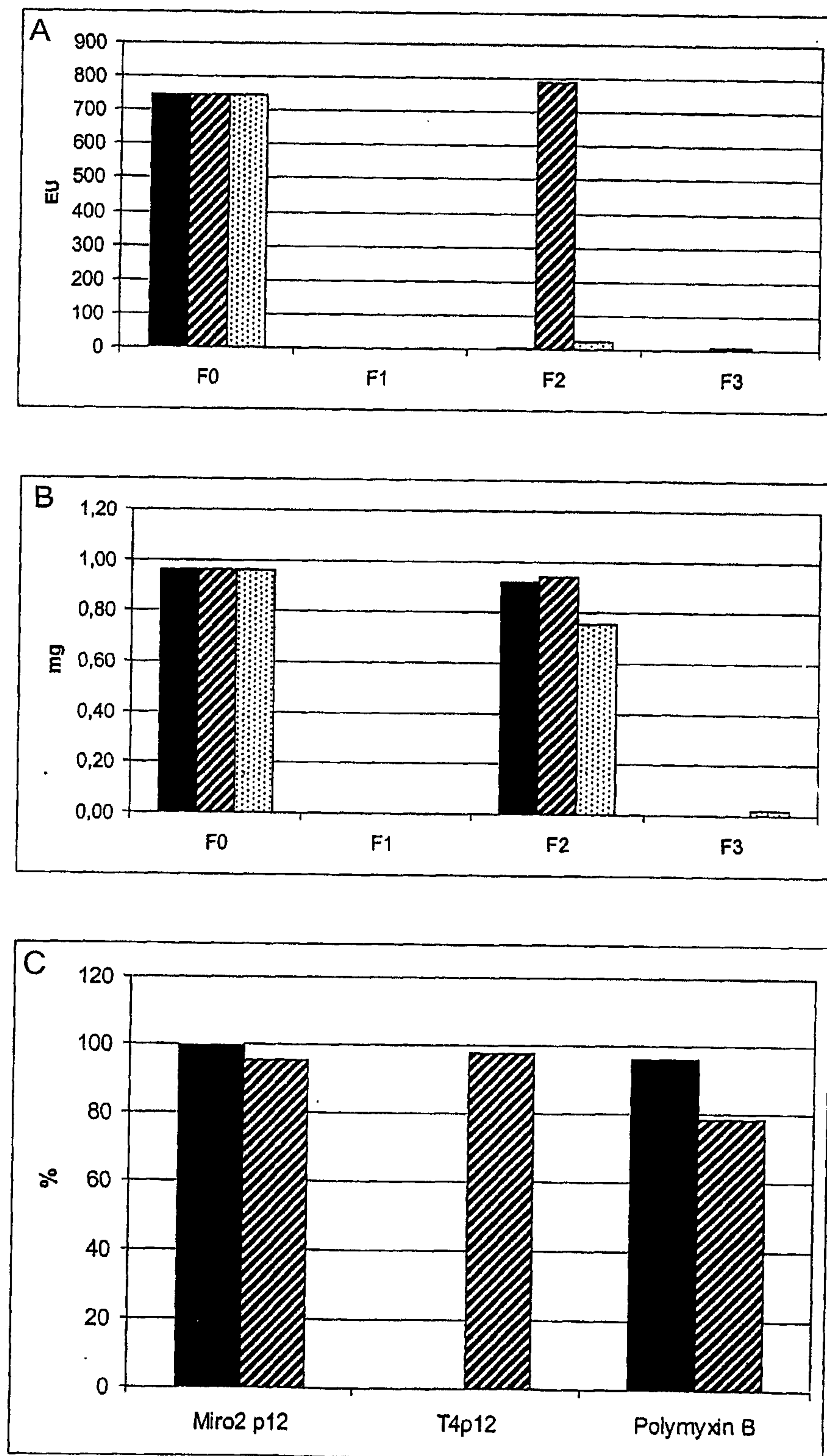


Fig. 5

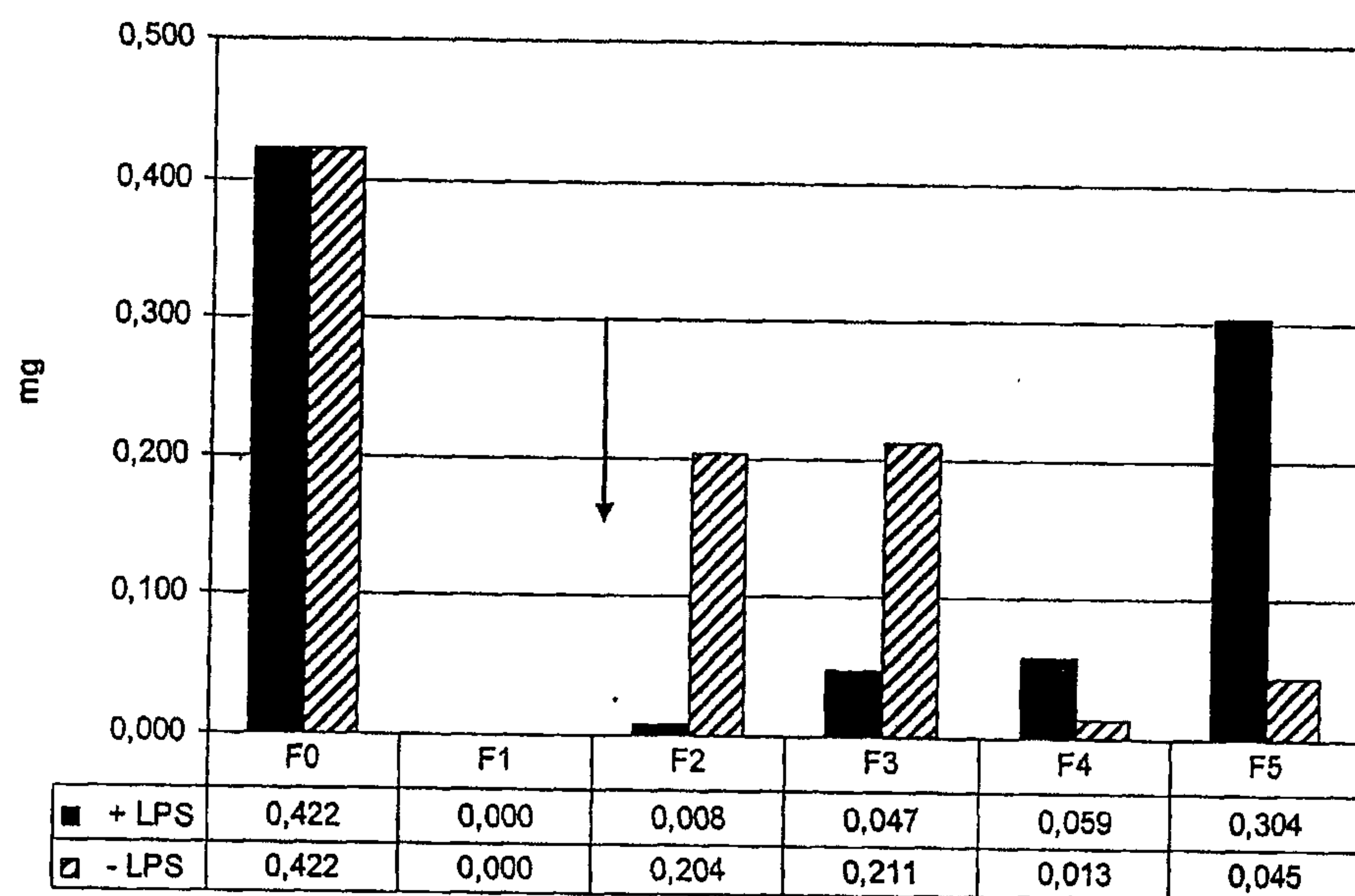


Fig. 6

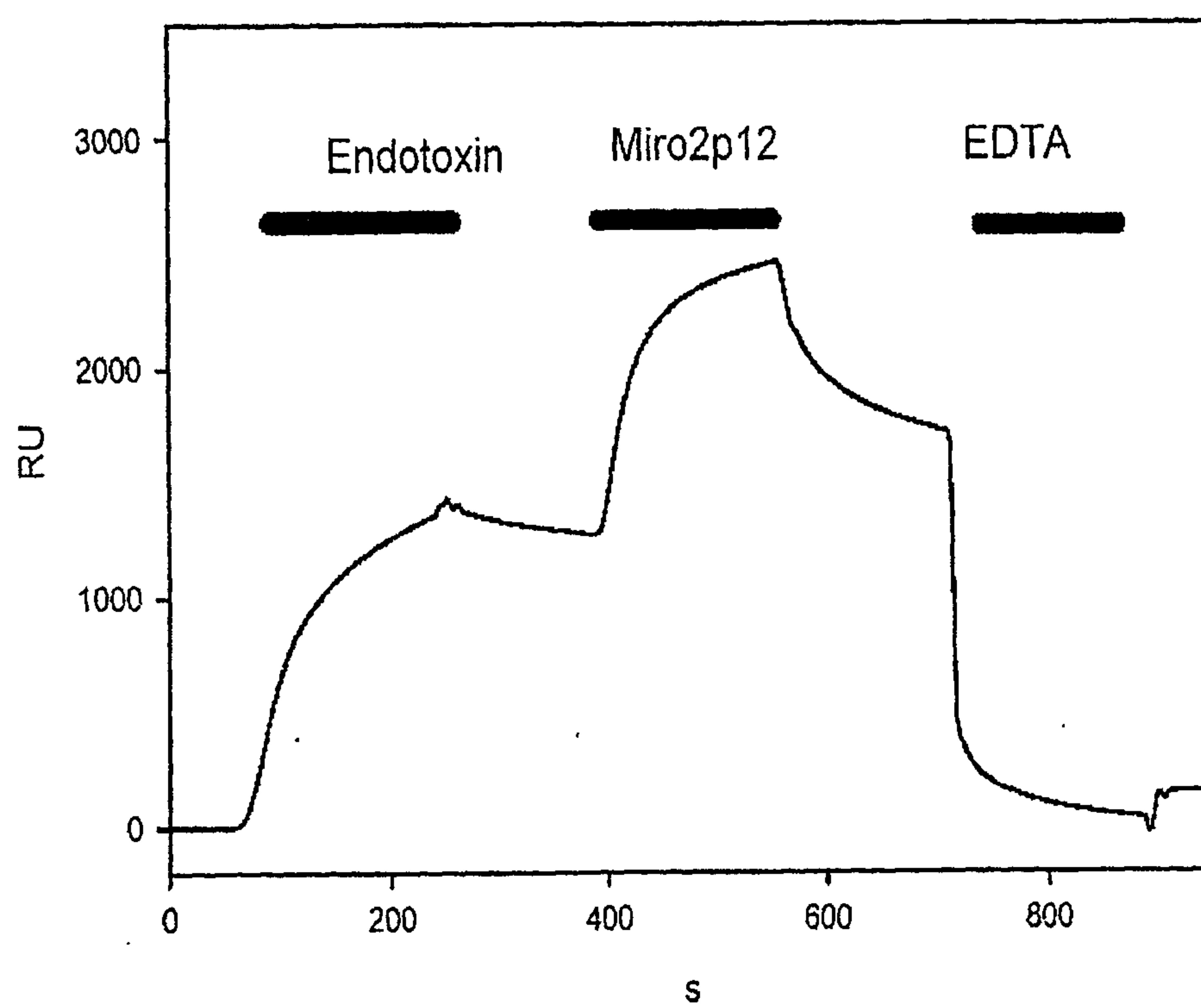


Fig. 7

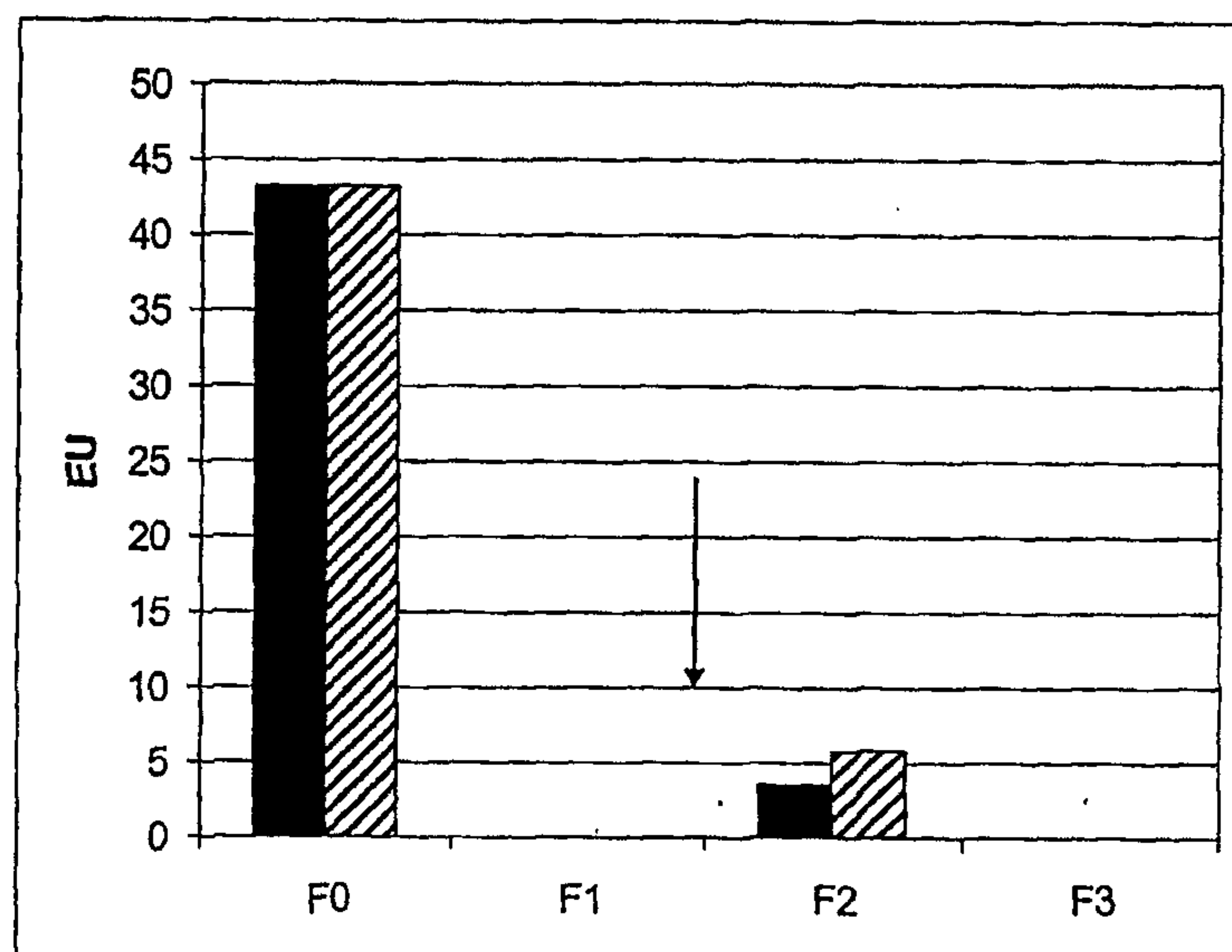


Fig. 8

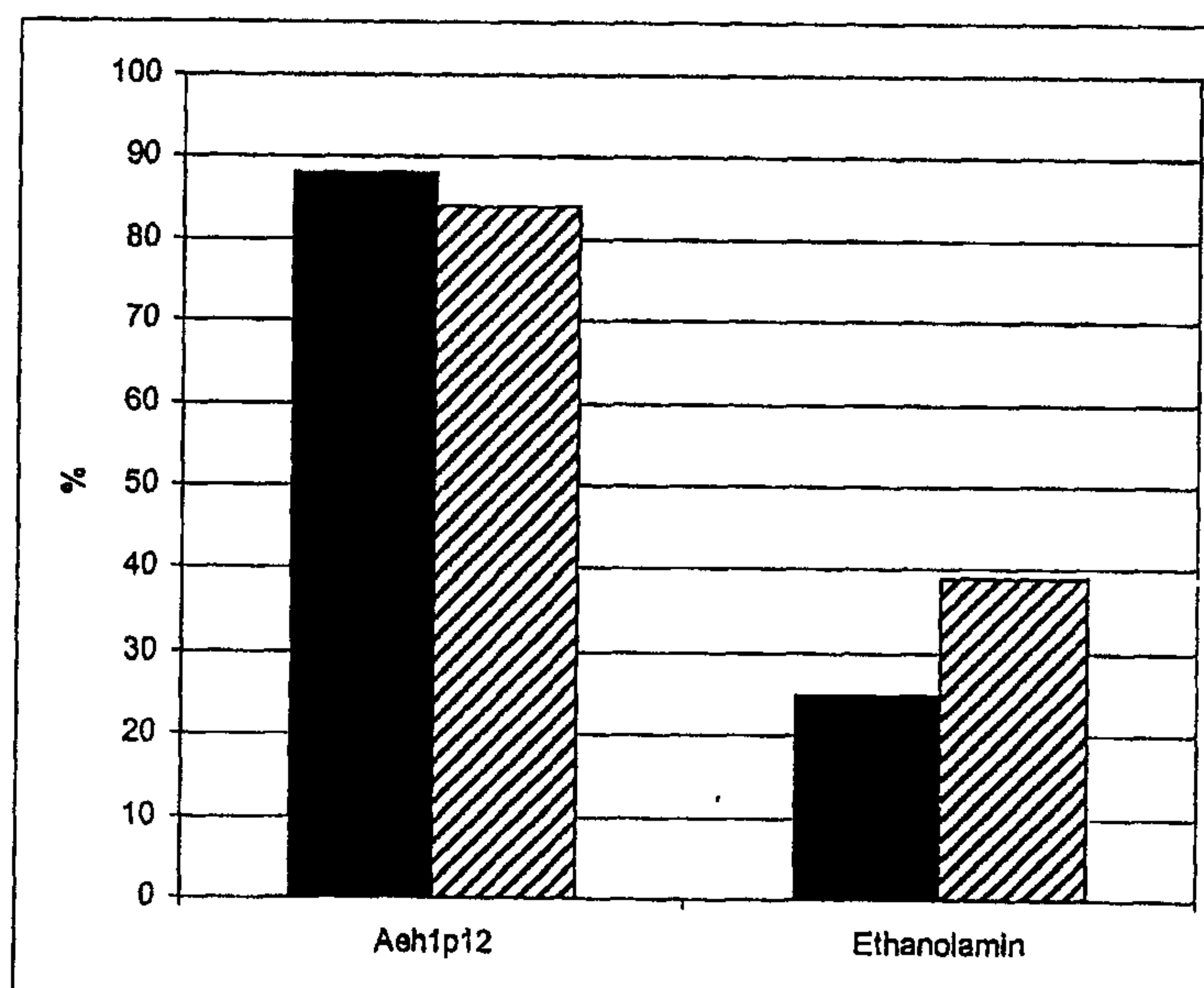


Fig. 9

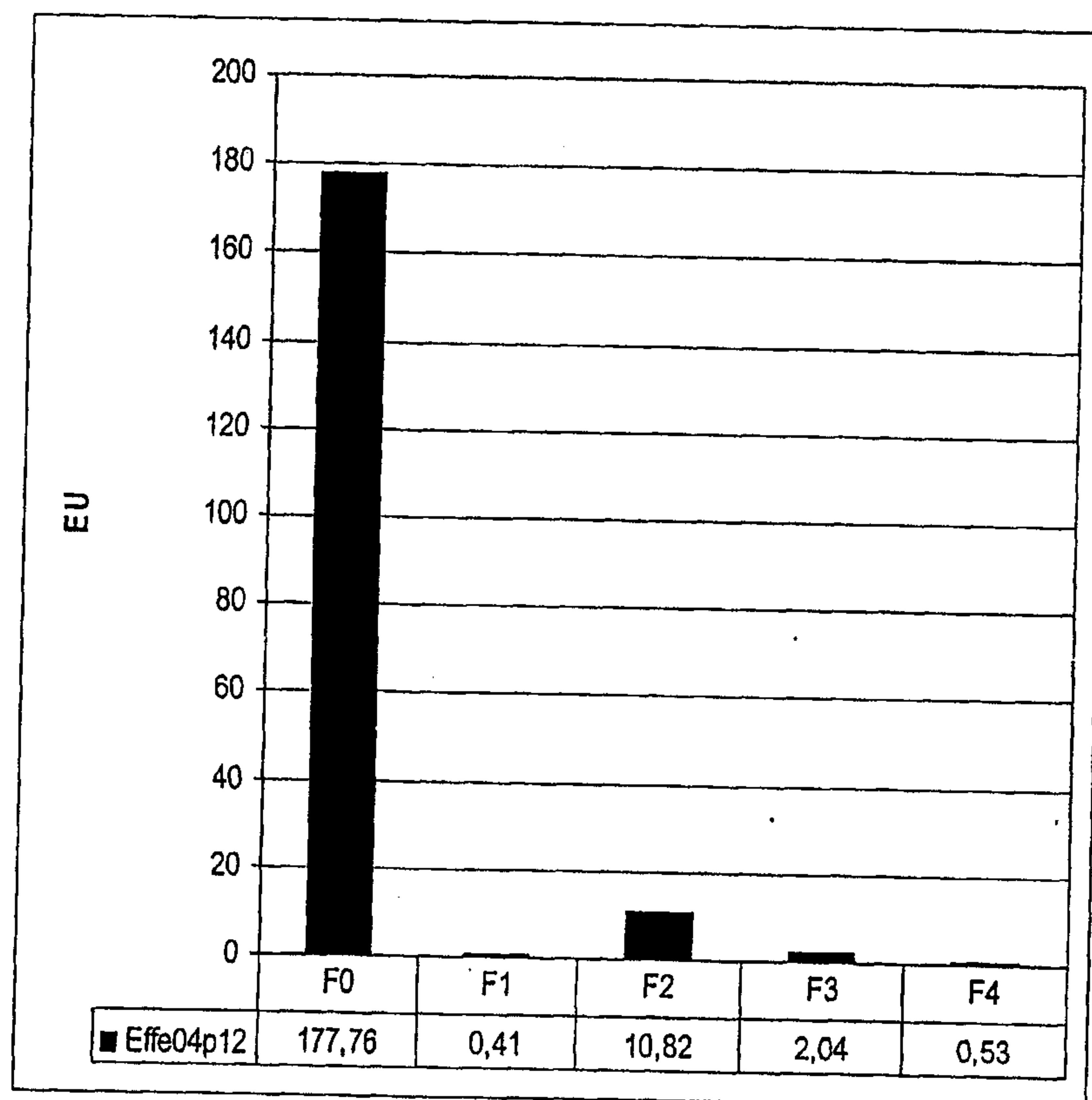
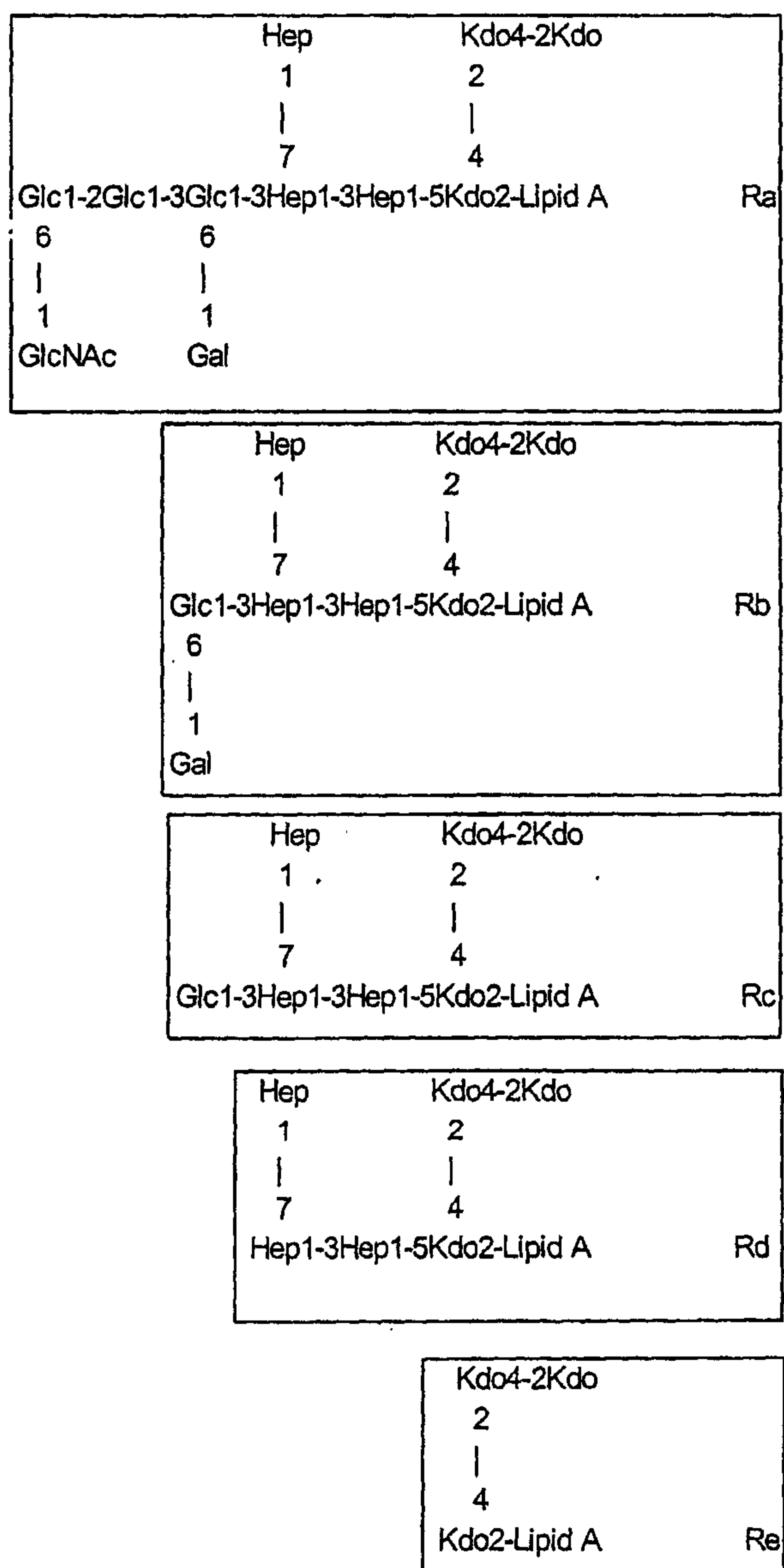


Fig. 10



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FIG.11 A

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K3p12      -----MSNNYQHVSNESRYVKFDPTDTNFPPEI---TDVQAAIAAISPAG
T2p12      -----MSNNYQHVSNESRYVKFDPTDTNFPPEI---TDVQAAIAAISPAG
T4p12      -----MSNNYQHVSNESRYVKFDPTDTNFPPEI---TDVHAAIAAISPAG
RB32-33    -----MSNNYQHVSNESKYVKFDPVGSNFPDTV---TTVQSALSKISNIG
AR1p12     -----MSNNYQHVSNESKYVKFDPVGSNFPDTV---TTVQSALSKISNIG
PP01p12    -----MSNNYQHVSNESKYVKFDPVGSNFPDTV---TTVQSALSKISNIG
RB69       -----MSNNYQHVSNESVYVEFDPTGSNFDSSI---TNVQAALASISAYG
Eff04_p12  MASASWSHPQFEKGASNNNTINHVS DKS IYVTFDPTGTDWPD TI---TNVQDALEKIGSWA
RB43p12    -----MSNNYQHVSNESVYVEFDPTGSNFDSSI---TNVQAALASISAYG
RB49p12    -----MANNTINHVS DKS IYVTFDPTGTDWPD TI---TNVQDALEKIGSWA
Miro1p12   ---MLEPPAVEKGAQQNNYHYS DLA KYTIFDPTNTQWPV AI---KDVQSALELIGSWA
Miro2p12   ---MASWSHPQFEKGAAQNNYHYS DLA KYTIFDPTNTQWPV AI---KDVQSALELIGSWA
44RR2      -----MVANNIKNHIS DSAES VNY---TGDNWP AIV---TTVADALDRVAPWA
PHG31p12   -----MVANNIKNHIS DSAES VNY---TGDNWP AIV---TTVADALDRVAPWA
AehIp12    -----MRTNNITQHIS DSAES VNY---TGDNWP AIV---TTVADALDRVAPWA
KVP40p12   -----MRTNNITQHIS DSAES VNY---TGDNWP AIV---TTVADALDRVAPWA
            :  *  .  :  .  .  .  :  :  .

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```

K3p12      VN--GVP---DASSTTKGILFLATEQEVIDGTNNTKAVTPATLATRLS-YPNATETVYGL
T2p12      VN--GVP---DASSTTKGILFLATEQEVIDGTNNTKAVTPATLATRLS-YPNATEAVYGL
T4p12      VN--GVP---DASSTTKGILFIPTQEVIDGTNNTKAVTPATLATRLS-YPNATETVYGL
RB32-33    VN--GIP---DATMEVKGIAMIASEQEVL DGTNNS KIVTPATLATRLS-YPNATETKYGL
AR1p12     VN--GIP---DATMEVKGIAMIASEQEVL DGTNNS KIVTPATLATRLS-YPNATETKYGL
PP01p12    VN--GIP---DASMEVKGIAMIASEQEVL DGTNNS KIVTPATLATRLS-YPNATETKYGL
RB69       VK--GVP---DASEAEKGV IQLATEQEVL DGFNST KAVTPATLNARLQ-YPNASETQYGV
Eff04_p12  RTDTGLP---IATTSVRGIAQIATEADINAGTDNTKIVTPKLLAYRMQ-NPKASQTVWGY
RB43p12    RTDVGLP---NAAPGMRGIAA IATEAMIDAGTDNETIVTPALLAYRLQ-NPHASQTVWGY
RB49p12    VN--GLP---DATEDTAGIAA IATEQEVL DGTNNS KIVTPKTLAVKMS-RPDATKEVKGI
Miro1p12   RTDTGLP---VASPTVAGVIRTATQAEVDAGTIGNAAVTPATLKSTVT-RPEATTAVLGL
Miro2p12   RTDTGLP---VASPTVAGVIRTATQAEVDAGTIGNAAVTPATLKSTVT-RPEATTAVLGL
44RR2      IIDNGLP---LATTQIAGIIRIATTAEMQAGTSANTAITPALLKLAME-TPQASETIVGN
PHG31p12   IIDNGLP---LATTQIAGIIRIATTAEMQAGTSANTAITPALLKLAME-TPQASETIVGN
AehIp12    TK--PLP---VASETVSGIAELATVDEVLLGNDKVRLVTPYTLQOKWA-RPNASDTVYGL
KVP40p12   VQ--PLPDYPVASTTVAGITKLSDEAAVVDPLNTDSAVTPSSLDYWMQN HATATELQYGF
            : *  * :  * :  .  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :

```

```

K3p12      TRYSTN---DEAIAGVNNESSITPAKFTVALNNAFETR-VSTESSNGVIKISSLPQALAG
T2p12      TRYSTD---DEAIAGVNNESSITPAKFTVALNNAFETR-VSTESSNGVIKISSLPQALAG
T4p12      TRYSTN---DEAIAGVNNESSITPAKFTVALNNAFETR-VSTESSNGVIKISSLPQALAG
RB32-33    TRYSTN---EETLEGS DNNSSITPQKLKYHTDDVFQNR-YSSSESSNGVIKISSTPAALAG
AR1p12     TRYSTN---EETLEGS DNNSSITPQKLKYHTDDVFQNR-YSSSESSNGVIKISSTPAALAG
PP01p12    TRYSTN---EETLEGS DNNSSITPQKLKYHTDDVFQNR-YSSSESSNGVIKISSTPAALAG
RB69       TKYATQ---EEAIAGTLDTVSITPLKLNQTDNTFSTR-YSTETTNGVIKIATQTAALAG
Eff04_p12  TKYSTD---AESTTVTNDASSITPR---SLNYVFNNR-KGTESVWGSSKIATTAQAVAG
RB43p12    TKYATD---AEAVDVANDLVSLTPR---SINVVFNNR-HASETVWGSSKLSTTAQATAG
RB49p12    TRFATM---EESLQESNENMAIGPD---TLNHVFTTK-KASESVQGTIKICSLEAAKIG
Miro1p12   TRYATN---TEAAALTAGNRTITAA---ALGHVFKTV-KAQENV DGTVRLTTAAQAQAG
Miro2p12   TRYATN---TEAAALTAGNRTITAA---ALGHVFKTV-KAQENV DGTVRLTTAAQAQAG
44RR2      TRYATN---AEALALT LNTAAITPAN---LGYVFANK-AATESARGTMRISTQAQATSG
PHG31p12   TRYATN---AEALALT LNTAAITPAN---LGYVFANK-AATESARGTMRISTQAQATSG
AehIp12    VRYN-----TVAEREEAAAKVDVTVNTASLWDVVRNKS IATESKRGSVSISTLVAAKAG
KVP40p12   VKLITESTIDTVAPSDPVEAAQKHAFTLKLNTALNTRFYATESDPGAVRLATNAQATT
            . :  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

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FIG. 11 B

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K3p12      --ADDTTAMTPLKTQQLAIKLIAPSETTATESDQGVVQLATVAQVRQGTREG-YAI
T2p12      --ADDTTAMTPLKTQQLAVKLIAPSKNAATESEQGVIQLATVAQARQGTREG-YAI
T4p12      --ADDTTAMTPLKTQQLAIKLIAPSETTATESDQGVVQLATVAQVRQGTREG-YAI
RB32-33    --VDDTTAMTPLKTQKLAIKLISQIAPSEDTASESVRGVVQLSTVAQTRQGTREG-YAI
AR1p12     --VDDTTAMTPLKTQKLAIKLISQIAPSEDTASESVRGVVQLSTVAQTRQGTREG-YAI
PP01p12    --VDDTTAMTPLKTQKLAIKLISQIAPSEDTASESVRGVVQLSTVAQTRQGTREG-YAI
RB69       --SDDTTAMTPLKTQQLAIKLISQIAPNNDPASESITGVVRLATVAQTRQGTREG-YAI
Eff04_p12  --TDNTVTMTPLKVKQAIASLVP---VQSSATESSQGLVQLATVAQVQAGTIREG-YAI
RB43p12    --TDDTTSMTPLKVKQAIASLVP---VQSNATESAFGLVQLATVSEVRAGTIRDG-FAI
RB49p12    --SDDTMAVTPPKMHTAIAQIVPGLIPDQNTATESAQGLVQLATNAQVLQGGIREG-FAI
Miro1p12   --TDETTAVTPKRVVEMIGKFSVS-PPSYTSATESNLGLVRVATQAQVAAGAVHDG-YAV
Miro2p12   --TDETTAVTPKRVVEMIGKFSVS-PPSYTSATESNLGLVRVATQAQVAAGAVHDG-YAV
44RR2      --TDDATTMTPLKTKLAIQALSQ---AWGTATESARGVVQMATVAQALQGTLRDG-FAI
PHG31p12   --TDDATTMTPLKTKLAIQALSQ---AWGTATESARGVVQMATVAQALQGTLRDG-FAI
AehIp12    --VDDTTAMTPAKVKAIDTFAVT---SVSGATETVTGTVKNSPALITNAALHTG--YAV
KVP40p12   GTLSTTVAMTPQVRKEMLDVWANT---TASDASETTKGLIRLANGTEVNSTLATEDNLAI
           . : : : * : : : * : : : * : : :

K3p12      SPYTFMNSSATEEYKGVIKLGTQSEVNSNN-ASVAVTGATLNNGRGSTTSMRGVVRLTTTA
T2p12      SPYTFMNSTATEEYKGVIKLGTQSEVNSNN-ASVAVTGATLNNGRGSTTSMRGVVKLTTTA
T4p12      SPYTFMNSSSTEEYKGVIKLGTQSEVNSNN-ASVAVTGATLNNGRGSTTSMRGVVKLTTTA
RB32-33    SPYTFMNSVATQEYKGVIRLGTQSEINSNL-GDVAVTGETLNNGRGATSSMRGVVKLTTQA
AR1p12     SPYTFMNSVATQEYKGVIRLGTQSEINSNL-GDVAVTGGTLNNGRGATGSMRGVVKLTTQA
PP01p12    SPYTFMNSVATQEYKGVIRLGTQSEINSNL-GDVAVTGETLNNGRGATGSMRGVVKLTTQA
RB69       SPYTFMNSVATQEYKGVIRLGTQAEINSNL-GDVAVTGETLNNGRGATGSMRGVVKLTTQA
Eff04_p12  SPYTFIRLTATESNLGVIRIASQTEANAGTDDTKAITAKKLINTRATGSQFGVVKLATTV
RB43p12    SPYTFIRLNATESDLGIVRLASQAEVNAGTDDTKAVTPLKLANLKGSGGSFGLVKLSTEV
RB49p12    SPYAFANARANENQAGTVKIASQSQMNAGSDDTVVVSARKFASTKATTSQYGVKLRDTV
Miro1p12   TPKTFMASKASDSVFGIVKFAKSDVASATSNNLAVTPKSLQALKSTKDKYGLTRLGSGSP
Miro2p12   TPKTFMASKASDSVFGIVKFAKSDVASATSNNLAVTPKSLQALKSTKDKYGLTRLGSGSP
44RR2      SPYTLSKMAGTESAAGMFKIASNSQILALADNTVVVTPAKLDILKATASQLGLVKLSGVS
PHG31p12   SPYTLSKMAGTESAAGMFKIASNSQILALADNTVVVTPAKLDILKATASQLGLVKLSGVS
AehIp12    TPKGFIETRAAQARVGTVRMATQAEANARTLGDVAISPATLPIASD--TOYGITALLHNA
KVP40p12   SPYRFNFRTATTTTRKAGFYLPDATVANARASNEHAVTVGTLNLFSSANSSRVGVAKIANNL
           : * : : : : : : : : : : : : : : : : : : : : : : : : : : :

K3p12      GSQSGGDASSALAWNADVHQGGQTINGTLRINNTLTIASGGANITGTVNMTGGYIQGK
T2p12      GSQSGGDASSALAWNADVHQGGQTINGTLRINNTLTIASGGANITGTVNMTGGYIQGK
T4p12      GSQSGGDASSALAWNADVHQGGQIIYGTLRIEDTFTIANGGANITGTVRMTGGYIQGN
RB32-33    GIAPEGDSSGALAWNADVINTRGGQTINGSLNLD---HLTANGIWSRGGMWKNG---DQ
AR1p12     GIAPEGDSSGALAWNADVINTRGGQTINGSLNLD---HLTANGIWSRGGMWKNG---DQ
PP01p12    GIAPEGDSSGALAWNADVINTRGGQTINGSLNLD---HLTANGIWSRGGMWKNG---DQ
RB69       GVAPEGDSSGALAWNADVINTRGGQTINGSLNLD---HLTANGIWSRGGMWKNG---DQ
Eff04_p12  GY---VANTALSSNAYVLPSPDR-----SAVINGSLEYEYSAIHNN-----K
RB43p12    NAG---LANTALSAGANVVPSNRD-----SAITGGALYQGSVAAAN-----K
RB49p12    GS---EANAALSANAKVLPSTG-----GTVSGNVYKGSNSDGN-----Q
Miro1p12   TT---DASLAAAATDAVEFKTRK-----INGKTLDNNDITITNNDINCYTR
Miro2p12   TT---DASLAAAATDAVEFKTRR-----INGKTLDNNDITITNNDINCYTR
44RR2      TA---AANTALAASAPVLYTSGG-----IITGDVTFGTGNMQGIQWSRNTD
PHG31p12   TA---AANTALAASAPVLYTSGG-----IITGDVTFGTGNMQGIQWSRNTD
AehIp12    QSG---VTNKALSAHGATLFINR-----NGDSMTGDLTVHNI FTANGQNGRGD
KVP40p12   TTN---DPLQALSAAMGYKLNEK-----IGDAGGTVTGTLKINNVQSVGGTQLMTN
           * :

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FIG.11 C

K3p12	RVVTQNEIDRTIPVGAIMMWAADSLPSDAWRFCCHGGTVSASDCPLYASRIGTRYGGSSSN
T2p12	RVVTQNEIDRTIPVGAIMMWAADSLPSDAWRFCCHGGTVSASDCPLYASRIGTRYGGTSSN
T4p12	RIVTQNEIDRTIPVGAIMMWAADSLPSDAWRFCCHGGTVSASDCPLYASRIGTRYGGNPSN
RB32-33	PVATERYASERVVPVGTIMMFAGDSAP-PGWIMCHGGTVSGDQYPDYRNTVGARFGGDWNN
AR1p12	PVATERYASERVVPVGTIMMFAGDSAP-PGWIMCHGGTVSGDQYPDYRNTVGTRFGGDWNN
PP01p12	PVATERYASERVVPVGTIMMFAGDSAP-PGWIMCHGGTVSGDQYPDYRNTVGTRFGGDWNN
RB69	PVATERYASERVVPVGTIQMFAGDSAP-PGWVLCCHGGTISGDQFPDYRNVVGTRFGGDWNN
Eff04_p12	YQWTDLDDWH-FPVGAIVMTGFQTDH-GSLYICDGRSLNKNNYPLLFERIGYTFGGGGDW
RB43p12	YQTHSDIEAS-LPIGCMMAAFNSDY-GNLCIANGRGMYTYEPELFALIGYTYGGSGNI
RB49p12	FVTKNELANHAMPIGGIILSGFNADR-GDFLICNGRSLNKNNYPLLFAIGYTFGGSGDN
Miro1p12	QESDGRYMPAGTRVGNVTWVEGQSWISRGATFTCNAPWEASSRLALNVNVKERNNDGYD
Miro2p12	QESDGRYMPAGTRVGNVTWVEGQSWISRGATFTCNAPWEASSRLALNVNVKERNNDGYD
44RR2	MAHIVFKNDSNADSNFSMQFCVGDDNNEYFRWVN--RFSGSDN----IMATLRPGGHMWL
PHG31p12	MAHIVFKNDSNADSNFSMQFCVGDDNNEYFRWVN--RFSGSDN----IMATLRPGGHMWL
AehIp12	SLTRKDYVDGLFNQ-KANISHTHGTPOESWTLIWQGFLDRGNF---VTNQPWWNFDAIV
KVP40p12	GLIESQAMLMYPVGSVYMSLVSTSPATLFGGTWARLAQGRVL---VSEGSYGGRTFAV
K3p12	PGLPDMRGLFVRGSGRGSHTLNPVNGNDQFGKPRLGVGCTGGYVGEVQKQMSYHKHAG
T2p12	PGLPDMRGLFVRGSGRGSHTLNPVNGNDQFGKPRLGVGCTGGYVGEVQKQMSYHKHAG
T4p12	PGLPDMRGLFVRGSGRGSHTLNPVNGNDQFGKPRLGVGCTGGYVGEVQIQQMSYHKHAG
RB32-33	PGIPDMRGLFVRGAGTGGHILNQ--RGQDGYGKDRGLGVGCDGMHVGGVQAQQMSYHKHAG
AR1p12	PGIPDMRGLFVRGAGTGGHILNQ--RGQDGYGKDRGLGVGCDGMHVGGVQAQQMSYHKHAG
PP01p12	PGIPDMRGLFVRGAGTGXHILNQ--RGQDGYGKDRGLGVGCDGMHVGGVQAQQISYHKHAG
RB69	PGIPDMRGLFVRGAGTGSHILNN--RGQDGYGKDRGLGVGCDGMHVGGVQAQQMSYHKHAG
Eff04_p12	FNIPDCRGVAVRGHGRGLNPN--RGYGTIEGDMLG-----WHEHPLQLIYQ--NGG
RB43p12	FNLPMRGVVARGFADAGRLDPG--RGFGTYQHHEVQ-----SHEHPLQMIYQ--SGG
RB49p12	FNLPMRGLVARGCDHGRNLDPG--RRFGSYQEDAMQR-----ITGKFPPVADRWRGWYGG
Miro1p12	NRIFRFV-VIVNGSQWGGEITLN-----IENTKGGRNGHSW
Miro2p12	NRIFRFV-VIVNGSQWGGEITLN-----IENTKGGRNGHSW
44RR2	AGNIDVNDFYIRSDRRLKHGFKPIENALDKIDLLNPG-----TYHKQYSLTDDRIV
PHG31p12	AGNIDVNDFYIRSDRRLKHGFKPIENALDKIDLLNPG-----TYHKQYSLTDDRIV
AehIp12	IESSRDGGSWFNTMEISRWQIEQ-----MQAKYPN---
KVP40p12	RQTGGEYEVQLTEATIPAHKHAG-----WGEHYDGNGI
K3p12	GFGEW---DDSGAFGNTRRSNFVGTTRKGLDWDNRSYFTNDGYEIDPASQRNSRYTLNRPE
T2p12	GFGHY---DDSGAFGNTRRSNFVGTTRKGLDWDNRSYFTNDGYEIDPASQRNSRYTLNRPE
T4p12	GFGEH---DDLGAFGNTRRSNFVGTTRKGLDWDNRSYFTNDGYEIDPESQRNSKYTLNRPE
RB32-33	GWGEY--QRHEAPFGASVYQGYLGTRKYSWDNRSYFTNDGFELG--GPRDALGTLNREG
AR1p12	GWGEY--NRSEGPFGASVYQGYLGTRKYSWDNRSYFTNDGFELG--GPRDALGTLNREG
PP01p12	AWGENGNNGRYAPFGASNGSGYLGNGRSADWDNHLFFTNDFGEMG--GPRDSFGTLNREG
RB69	GWGEF--QRHEAPFGASVYQGYLGTRKYSWDNRSYFTNDGFELG--GHRDATGTLNREG
Eff04_p12	NIPKW-----QAVYELKSAEKNDQSARVFDASITKATGVG-----
RB43p12	NLPSW-----QCVYELRTAEKNDQQLYWPDPSSLKAMAVG-----
RB49p12	AFTAQ-----RGQWSTNYKNGGGDDWGTTVNFDSGRSVRT-----
Miro1p12	RFEAY-----ASSNFFFNIPPNATVQIRPTEDS-----
Miro2p12	RFEAY-----ASSNFFFNIPPNATVQIRPTEDS-----
44RR2	GLEAG-----IFAQDFQKAMPEGVRSLEDGTLTVSPMG-----AIA
PHG31p12	GLEAG-----IFAQDFQKAMPEGVRSLEDGTLTVSPMG-----AIA
AehIp12	-FNLVS-----AQEYYWFGKFRADG---MYFDT-----
KVP40p12	GFGVAK-----QYGRNNPGSRRTDSDNYLYTSPVGGNQ-----

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FIG.11 D

K3p12	LIGNETRPWNISLNYIIKVKE
T2p12	LIGNETRPWNISLNYIIKVKE
T4p12	LIGNETRPWNISLNYIIKVKE
RB32-33	LIGYETRPWNISLNYIIKIHY
AR1p12	LIGYETRPWNISLNYIIKIHY
PP01p12	LIGYETRPWNISLNYIIKIHY
RB69	LIGYETRPWNISLNYIIKVHY
Eff04_p12	--GEETRMKNIALNYVIRVL-
RB43p12	--GNETRMKNLAINYVIRVR-
RB49p12	--ANETRVKSLALNYIIRVR-
Mirolp12	----RIIFYDCMLTFCTNRP-
Miro2p12	----RIIFYDCMLTFCTNRP-
44RR2	FLIQCNKELKARLEKLEGIK-
PHG31p12	FLIQCNKELKARLEKLEGIK-
Aeh1p12	---HTENCYLWRIYGVNKTWS
KVP40p12	--AHNNVQPYYTVYMWERTA-

:

Fig.12

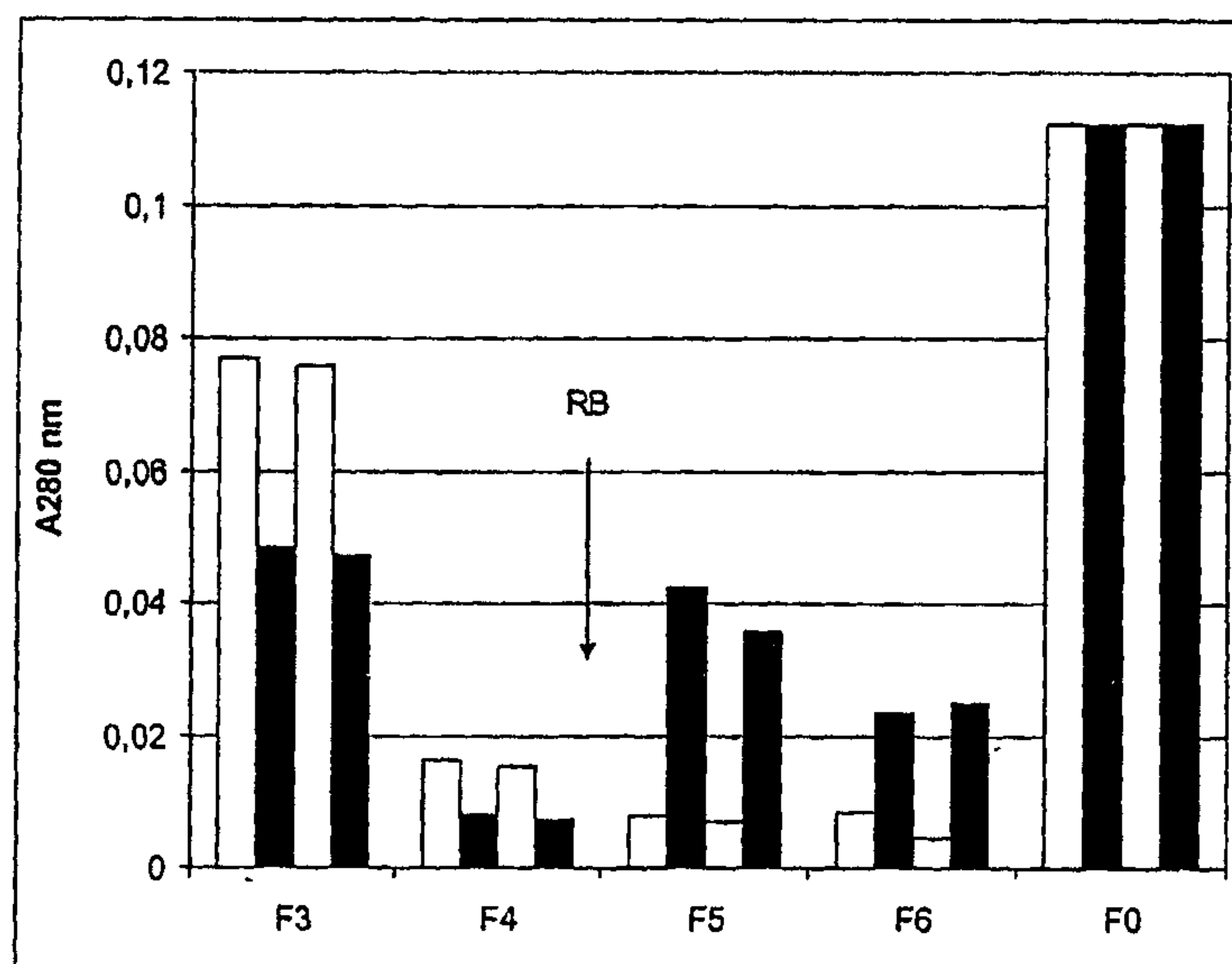


Fig. 13

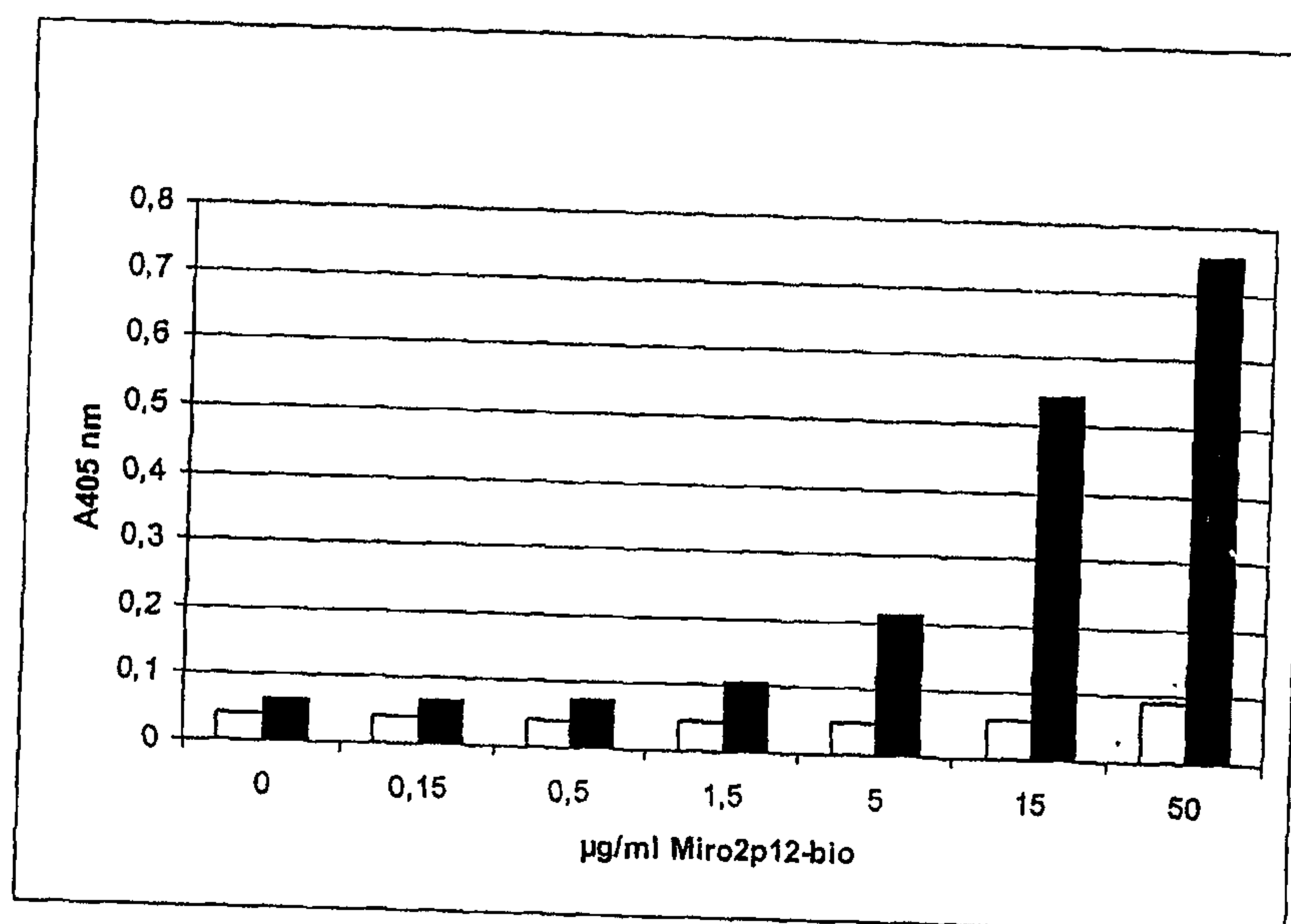


Fig. 14

