



(22) Date de dépôt/Filing Date: 1998/01/27

(41) Mise à la disp. pub./Open to Public Insp.: 1998/07/28

(45) Date de délivrance/Issue Date: 2002/07/09

(30) Priorité/Priority: 1997/01/28 (9700244-8) SE

(51) Cl.Int.⁶/Int.Cl.⁶ C07K 14/435, A61K 38/47, A61K 38/17

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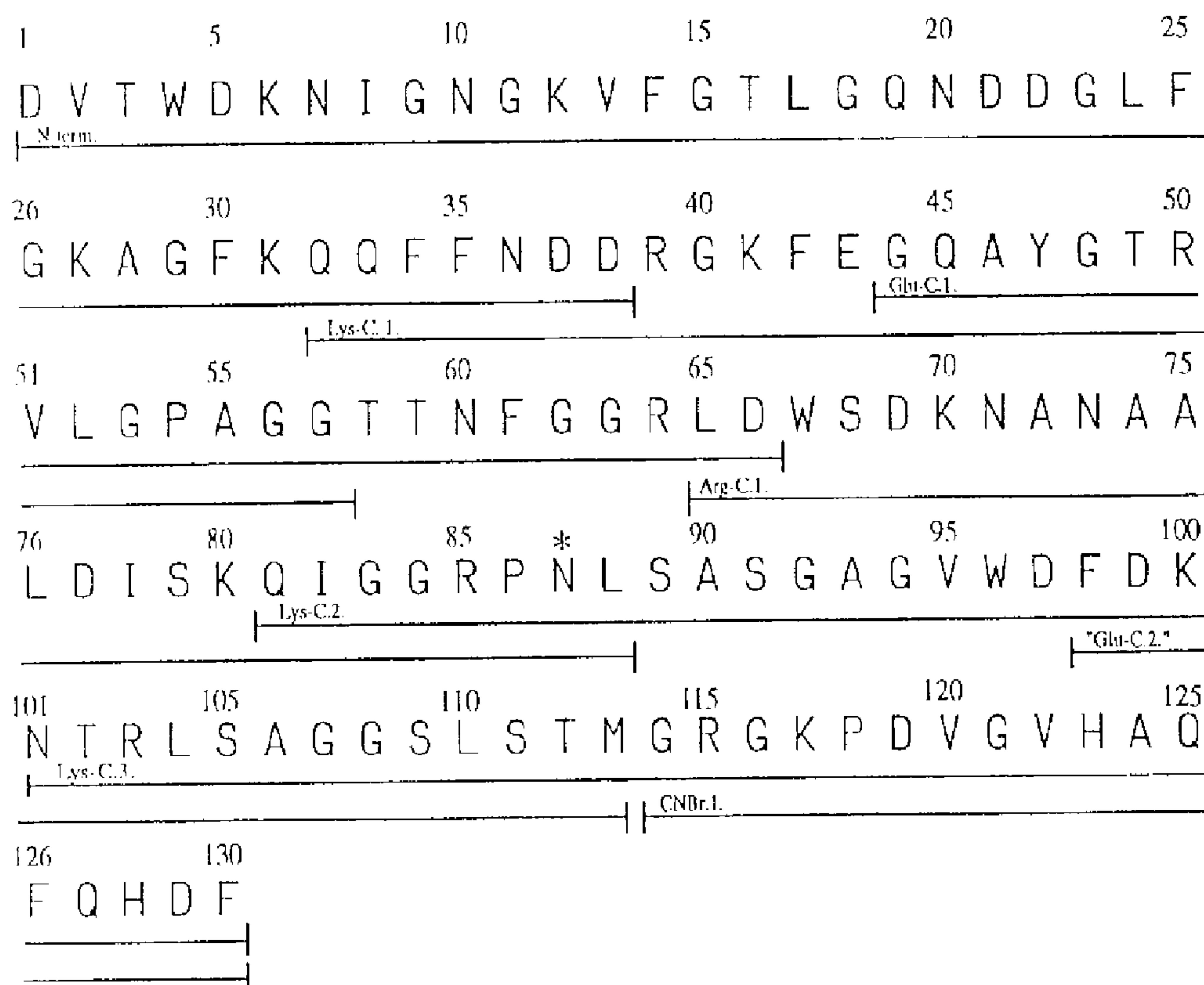
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(54) Title: NOVEL ANTIBACTERIAL PROTEIN



(57) Abrégé/Abstract:

The present invention relates to a novel antibacterial protein, called gloverin. Preferably gloverin is isolated from *Hyalophora* moths. Alternatively, gloverin is produced by genetic engineering or by chemical synthesis. Also, the invention relates to pharmaceutical compositions comprising gloverin or pharmaceutically active fragments thereof and use of gloverin or fragments thereof as a medicament against bacterial infection. Furthermore, the invention relates to a method of treating bacterial infection comprising administration of gloverin or pharmaceutically active fragments thereof.



63786-116

ABSTRACT

The present invention relates to a novel antibacterial protein, called gloverin. Preferably gloverin is isolated from *Hyalophora* moths. Alternatively, gloverin is produced by genetic engineering or by chemical synthesis. Also, the invention relates to pharmaceutical compositions comprising gloverin or pharmaceutically active fragments thereof and use of gloverin or fragments thereof as a medicament against bacterial infection. Furthermore, the invention relates to a method of treating bacterial infection comprising administration of gloverin or pharmaceutically active fragments thereof.

Technical field

The present invention relates to a novel antibacterial protein, designated gloverin, representing a new class of antibacterial insect proteins which can be isolated from *Lepidoptera*, more specifically the pupa of *Hyalophora* giant silk moths.

Furthermore, the invention relates to pharmaceutical compositions comprising gloverin as a medicament and use thereof in a method against bacterial infection.

Background of the invention

Infection of pupae of lepidopterans with live non-pathogenic bacteria induces the synthesis of a variety of antibacterial polypeptides secreted into the hemolymph. Previous studies have identified three main classes of antibacterial proteins namely lysozyme, cecropins and attacins [1]. Lysozyme [2,3] attacks the cell wall of gram-positive bacteria. The small (4-5 kDa), cationic cecropins [3,4] display a strong bactericidal effect against a variety of gram-positive and gram-negative bacteria. The attacins [5,6,7] (20 kDa) exist in two forms; one basic (pI=9) and one neutral pI=7) and the antibacterial effect is directed only against gram-negative bacteria.

Several forms of these antibacterial proteins have been found in various insect species. Peptides related to cecropins can be found not only in insects but also in vertebrates [8]. The same is true for the ubiquitous lysozymes. A protein related to the attacins, sarcotoxin IIA, has been found in the dipteran *Sarcophaga* [9].

Another class of antibacterial proteins from insects is the insect defensins [10]. They are characterised by an amino acid sequence of 38 to 43 amino acids containing six cysteines, forming three disulphide bridges. Different variants of insect defensins have been found in several insect species. Other related insect proteins are the dipterocins with a molecular mass of 8.6 kDa that are effective against gram-negative bacteria [11] and the hemolins that belong to

the immunoglobulin superfamily and are suggested to play a role in the regulation of cell adhesion during the cellular response to bacterial infections [12,13].

In addition to the antibacterial proteins from insects, there is also a number of antibacterial proteins isolated from mammals e.g. the bactericidal/permeability increasing protein (BPI) [14,15] and the defensins [16]. The mammalian defensins differ structurally from insect defensins, although they have similar size and charge.

Summary of the invention

The present invention provides a novel antibacterial protein, called gloverin. Gloverin is a basic (with a pI of about 9) protein with a molecular weight of about 14 kD containing a large number of glycine residues but no cysteine. Gloverin displays no strong sequence similarity to other known proteins. Gloverin inhibits the growth of gram-negative bacteria, such as *Escherichia coli*. The minimal concentration required for inhibition of bacterial growth is 1-3 μ M, which is less than 5% of the concentration of gloverin in the hemolymph of infected pupae. The synthesis of vital outer membrane proteins and, consequently, the permeability of the outer membrane are affected, indicating that the activity of gloverin is directed to the outer membrane of gram-negative bacteria.

Preferably, the novel antibacterial protein, gloverin, according to the invention is isolated from *Hyalophora* moths. Alternatively, gloverin is produced by genetic engineering or by chemical synthesis.

Also, the invention relates to pharmaceutical compositions comprising gloverin or pharmaceutically active fragments thereof and use of gloverin or fragments thereof as a medicament against bacterial infection. Furthermore, the invention relates to a method of treating bacterial infection comprising administration of gloverin or fragments thereof.

63786-116

3

Detailed description of the invention

MATERIALS AND METHODS

Isolation of protein. Diapausing pupae of *Hyalophora gloveri* were injected with 105 live *Enterobacter cloacae* β 12. After 7 days the hemolymph was collected as previously described [3]. Gloverin was purified from freshly collected or frozen hemolymph by the following procedure: 50 ml hemolymph was diluted five times with ice-cold distilled water and centrifuged for 10 min at 17000xg, 4 °C. Saturated ammonium sulphate (SAS) solution was added to the supernatant to give 25 % SAS final concentration. After 30 min at room temperature the precipitate was collected by centrifugation for 10 min at 17000xg, 4°C. The precipitate was dissolved in 10 ml distilled water and desalted on a Sephadex^{*} G-25, PD-10 column (Pharmacia, Sweden) equilibrated with the starting buffer used in the subsequent ion-exchange chromatography step. This was performed on a DEAE-Sepharose^{*} CL-6B column (3 x 6 cm)(Pharmacia, Sweden) equilibrated with 20 mM diaminopropane, adjusted to pH 10.1 with hydrochloric acid, at room temperature. Proteins were eluted using a gradient of 1 M sodium chloride in starting buffer. A subsequent gel filtration step was performed on a Superdex^{*}-75 column (1x30cm) (Pharmacia, Sweden) equilibrated with 0.1 M ammoniumbicarbonate.

Approximately 1.5 mg of purified gloverin were recovered from 50 ml of hemolymph collected from 50 pupae. The purity of the isolated protein was ascertained by sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry as described below.

Electrophoresis. SDS-PAGE was performed in 12.5% (w/v) slab gels by the method of Laemmli [17] but with a 4.5% stacking gel, containing 9% glycerol. Isoelectric focusing was performed using a Phast^{*} system (Pharmacia, Sweden) following the manufacturers standard protocols.

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63786-116

4

Automated amino acid sequence analysis [18] was performed using an ABI 477A (Applied Biosystems) protein sequencer with an on-line ABI 120A PTH analyser following standard protocols.

Gloverin was cleaved using cyanogen bromide, Glu-C, Lys-C or Arg-C endoproteinase (Boehringer Mannheim). Following cleavage with cyanogen bromide or Glu-C endoproteinase the digest was separated on a Superdex* - 75 gel filtration column in 0.1 M ammoniumbicarbonate. When cleaved with Lys-C or Arg-C endoproteinase the digest was separated by RP-HPLC on a Brownlee* C-18, 5 m column, 2.1x30 mm, eluted with a gradient of 0 - 70% acetonitrile in water containing 0.1% trifluoroacetic acid during 60 min with a flow rate of 0.3 ml/min.

Chromatography was carried out using an FPLC system (Pharmacia, Sweden). The effluent was monitored at 214 nm. All fractions collected were analysed by mass spectrometry.

Sequence comparison. The databases Swiss protein (release 27.0), and PIR protein (release 35.0) were searched by the program FASTA [19] using the Genetic computer group software [20].

Amino acid analysis. Amino acid analyses were performed by the ion exchange ninhydrin method.

Mass spectrometry. Plasma desorption mass spectra for cleavage products during sequence work were obtained using a BIOION 20 mass spectrometer (Applied Biosystems).

Circular dichroism. Circular dichroism (CD) measurements were performed on a Jasco*41A spectropolarimeter. d-10 Camphor-sulphonic acid was used for calibration with D_e taken as +2.37 at 290 nm. All spectra were recorded at 25°C using a 0.1 cm cell. Protein concentrations used were 0.1 mg/ml for estimating the optimum concentration of hexafluoro-iso-propanol and 0.3 mg/ml for recording the complete spectra, respectively.

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Protein concentrations were determined spectrophotometrically at 280 nm using the absorptive value of 18 350 M⁻¹cm⁻¹. The mean residue ellipticity expressed in deg.cm/dmol was calculated at every nm and is given as the average of two analyses. The mean residue weight used was 106.4 g/mol.

NMR-analyses. 1D ¹H-NMR analyses were performed on a Varian* 400 MHz FT NMR spectrometer.

Ultracentrifugation. Equilibrium and sedimentation experiments were performed using an Optima XL-A (Beckman Inc.) analytical ultracentrifuge.

Bacterial strains. D21f2 [21] is a *rfa* mutant of the *E. coli* K-12 strain D21 [22], with a deep rough, heptose-less lipopolysaccharide (LPS) (= chemotype Re). The gram-positive strain used was *Bacillus megaterium* Bm 11 [23]. The term "deep rough" used herein means that the LPS chain is shortened.

Antibacterial assay. The antibacterial activity of purified gloverin was assayed by recording the growth of liquid cultures in microtiter plates (NUNC, Denmark), 200 µl/well. Gloverin was added to LB medium at 5 - 10 µM final concentration and this mixture was inoculated with 5x10⁶ cells in mid-log phase. The cultures were incubated at 37°C on a rotary shaker and growth was recorded every 20 min by monitoring the absorbance at 560 nm with a Titertek Multiskan* spectrophotometer.

In some experiments samples were withdrawn from the growing cultures at different times and spread on LB agar plates to determine the correlation between number of viable cells and absorbance.

Radioactive labelling of bacterial proteins. Cells were grown as described above for the antibacterial assay except that LB was substituted with M9 minimal medium supplemented with 0.4% (w/v) glucose and amino acids, except methionine. L-[³⁵S] methionine (>37 TBq/mmol; Amersham, UK) was added to the cultures after 2 h, to a final concentration of 25 µCi/ml. Labelling

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63786-116

6

was continued for 10 min and then stopped by the addition of trichloroacetic acid to a final concentration of 10%(w/v). The labelled and precipitated cells were analysed on SDS-PAGE and the dried gels were overlaid with Kodak* X-omat AR-film and exposed for two days at room temperature.

The invention will now be described below with reference to the accompanying drawings, in which:

Fig. 1 represents SDS PAGE analysis of SDS-precipitated hemolymph and purified proteins.

Lanes: (1) Non-immune hemolymph (2) immune hemolymph at day 7 (3) purified gloverin (4) purified basic attacin (5) purified neutral attacin

Fig. 2 The amino acid sequence of gloverin.

The peptides used for sequencing are underlined to show overlaps. The peptide obtained by cleavage with cyanogenbromide is designated "CNBr.1." The peptides obtained by digestion with endoproteinase Glu-C, Arg-C and Lys-C are designated "Glu-C.1-2.", "Arg-C.1. and "Lys-C.1-3.", respectively. A potential glycosylation site is indicated by *.

Fig. 3 Laser desorption mass spectrum for gloverin.

Fig.4 Circular dichroism spectrum for gloverin.

Gloverin dissolved in 10 mM phosphate pH 6.4 (1) and with the addition of 20%(v/v) hexafluoro-iso-propanol (2).

Fig. 5 Effect of gloverin on the growth of D21f2.

Gloverin was added at time zero at a concentration of 5mM (1) or 10mM (2). The control (C) represents growth in the absence of gloverin. Panel A shows the optical density of the growing cultures. Panel B shows the number of viable cells in samples withdrawn from the cultures at times indicated.

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63786-116

7

Fig. 6 Effect of Triton X-100^{*} and lysozyme on D21f2 treated with gloverin. Triton X-100 (final concentration; 1%(w/v) (panel A); or chicken lysozyme (final concentration; 200mg/ml) (panel B); was added at time indicated by arrow to cultures grown in the absence (1) or presence (3) of gloverin (5 μ M). Curves (2) represent growth in the presence of gloverin (5 μ M) only, and curves (C) are controls without any additions.

Fig. 7 Effect of lipopolysaccharide (LPS) and magnesium on the growth-inhibiting activity of gloverin on D21f2.

In panel A curve (1) represents growth with the addition of 50 μ M of LPS. Curve (2) and (3) represent growth with the addition of 10 μ M of gloverin and 50 μ M or 30 μ M of LPS, respectively. Curve (4) represents growth with the addition of 10 μ M of gloverin solely and curve (C) represents the growth of D21f2 without any additives. In panel B curve (1) represents growth of D21f2 in the presence of 10 μ M of gloverin and 40 mM MgCl₂. Curve (2) shows growth in 10 μ M gloverin only and (C) represents the growth of D21f2 without any additives.

The addition of 40 mM MgCl₂ to the control culture has no effect (not shown).

Fig. 8 Autoradiogram of SDS-PAGE showing the effect of gloverin on synthesis of the outer membrane proteins Omp F/C and Omp A in 35S-methionine labelled D21f2 cells.

Lanes: (1) Control, untreated bacteria; (2) Bacteria incubated with gloverin (10 μ M) for 2 h

RESULTS

Isolation of protein. Ion-exchange chromatography of ammonium sulphate precipitated immune hemolymph resulted in two large peaks as determined at 280 nm. Analysis by SDS-PAGE showed that the first eluted of these consisted of gloverin and the basic form of attacina, while the second peak contained the neutral form of attacina and some additional proteins (data not shown). In order to further separate gloverin from attacina the gloverin containing peak from the

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ion-exchanger was applied on a Superdex 75 column which yielded gloverin free of attacin.

The SDS-PAGE analysis (Fig. 1) of purified proteins and the hemolymph from immunised and non-immunised pupae demonstrates that gloverin is induced by infection. Isoelectric focusing showed that the purified gloverin has an isoelectric point of 8.5 (data not shown).

Sequence analysis. The amino acid sequence of the above described gloverin is shown in Fig. 2, which also includes the sequence of the different cleavage fragments used. One digestion with Glu-C endoprotease was by accident performed without sufficient buffering causing the enzyme to cleave after both glutamic acid and aspartic acid. This produced the peptide from amino acid 98 to 113 and gave an overlapping sequence in the region of amino acid number 100. Comparison of the sequence of gloverin with other sequences in current data banks revealed no proteins with strong sequence similarities.

With knowledge of the amino acid sequence it is possible to produce gloverin by chemical synthesis. The invention relates to gloverin and gloverin-like sequences. The main criterion is that the specific gloverin-activity is retained in the protein/fragment.

It is realized by the skilled man in the art that the DNA sequence encoding gloverin can be obtained from the above information. Thus, the invention also encompasses DNA sequences encoding gloverin and gloverin-like proteins. Furthermore, the invention relates to such proteins produced by conventional genetic engineering.

Amino acid analysis. The result of the amino acid analysis of gloverin is presented in Table 1 and is compared with the composition deduced from the sequence. Included is also the amino acid composition for the corresponding protein isolated from *Hyalophora cecropia*.

63786-116

9

Table 1.
Amino acid composition for gloverin

Amino acid	Amino acid composition for gloverin from <i>Hyalophora gloveri</i> according to sequence	Amino acid composition for gloverin from <i>Hyalophora gloveri</i> according to a.a- analysis	Amino acid composition for gloverin from <i>Hyalophora cecropia</i> according to a.a- analysis
Ala	10	9,8	10,3
Arg	6	5,8	5,1
Asn	9		
Asp	13	20,5	20,1
Cys	0	0	0,8
Gln	7		
Glu	1	7,3	7,7
Gly	24	22,5	21,7
His	2	2,1	2,0
Ile	3	3,2	3,5
Leu	8	8,0	7,8
Lys	9	7,9	8,7
Met	1	1,1	1,2
Phe	10	9,7	9,4
Pro	3	3,3	3,8
Ser	7	7,2	7,4
Thr	7	6,8	7,3
Trp	3	-	-
Tyr	1	1,1	1,7
Val	6	5,8	6,2

Mass spectrometry. Laser desorption mass spectra of gloverin (Fig. 3) indicated a molecular mass of 13786 Da, which is in good agreement with the value of 13785 Da as calculated from the amino acid sequence. There is no

indication that gloverin is glycosylated, although there is a potential glycosylation site at asparagine 87.

Conformational studies. The CD-spectrum of gloverin in 10 mM phosphate, pH 6.4, can be interpreted as a reflection of a mainly random-coil structure (Fig. 4). To estimate the possible structure present in a more hydrophobic, membrane-like environment, CD-spectra were recorded in different concentrations of hexafluoro-iso-propanol. In a hydrophobic environment the spectrum changes to reflect a conformation having large amounts (approx. 50%) of alpha-helix structure (Fig. 4). The degree of assumed alpha-helix reaches a maximum at a concentration of 20 % of hexafluoro-iso-propanol. The result from the NMR-analysis confirms the conformational change that was indicated by the CD experiments (data not shown).

From the ultracentrifugation sedimentation experiments of gloverin in 10 mM phosphate, pH 6.4, the following parameters were calculated: sedimentation coefficient (S_{20}^w) = 1.4 S, diffusion coefficient (D) = $8.95 \times 10^{-7} \text{ cm}^2\text{s}^{-1}$ and a friction ratio (f/f_0) = 1.5. These values are in accordance with the expected values to be obtained for a protein of estimated molecular weight of 13.8 kDa and present in an extended conformation.

The equilibrium experiments gave a molecular weight of 13.8 kDa showing that the protein exists as a monomer in water solution.

Antibacterial activity.

The growth of *E. coli* K-12 is inhibited. Addition of gloverin to growing cultures of sensitive *E. coli* caused a decrease in the growth rate. This effect was noticeable after 1 h. After 2 - 3 h, growth was completely inhibited (Fig. 5) and prolonged exposure to gloverin resulted in a decrease in cell density. The remaining cells were still viable since the cultures recovered and continued to grow when incubated over night (data not shown).

Included in Fig. 5 is also a viable count experiment showing the correlation between cell density and the number of viable cells. No inhibitory effect of gloverin on the gram-positive cell *Bacillus megaterum* could be observed, using

concentrations of up to 100 mM of gloverin (not shown). The growth-inhibiting effect of gloverin is not significantly affected by heating the protein to 100°C for 10 min (data not shown).

The permeability of the outer membrane increases. Addition of the non-ionic detergent Triton X-100 to a culture of *E. coli* D21f2 grown for 2.2 h in the presence of gloverin resulted in a drastic drop in absorbance, in contrast to the much smaller effect of Triton X-100 on untreated control cultures (Fig. 6). The sensitivity to lysozyme was also increased by gloverin-treatment (Fig. 6). These results suggest that gloverin affects the integrity of the outer membrane, allowing entry of substances that are normally excluded by this permeability barrier, such as conventional antibiotics. Combined therapy with gloverin and conventional antibiotic(s) will lower the dose normally required for the antibiotic(s).

Mg²⁺ inhibits the activity of gloverin. The effect of gloverin on the growth of D21f2 was inhibited in the presence of 40 mM Mg²⁺ (Fig. 7B). This result is in accordance with the role of magnesium in stabilising the outer membrane. Binding to free LPS inhibits activity. Pre-incubation of gloverin with soluble LPS (Rd)(Sigma) for 30 min at 37°C prior to addition of the mixture to a growing culture of D21f2 cells blocks the antibacterial effect of gloverin (Fig. 7A). The inhibitory effect of LPS is concentration-dependent.

The synthesis of outer membrane proteins is affected. SDS-PAGE analysis of the protein content of gloverin-treated and radioactively labelled D21f2 cells showed that there was no general effect on protein synthesis. However, gloverin caused a specific inhibition of the synthesis of the outer membrane proteins Omp F, Omp C and Omp A. Some additional, unidentified proteins were also affected (Fig. 8).

DISCUSSION

A novel, antibacterial protein isolated from the immune hemolymph of *Hyalophora gloveri* pupae, is described in functional and structural terms.

The studied gloverin has a molecular mass of 13785 Da and consists of 130 amino acids without any cysteines but with a high content (18.5%) of glycine. Ultracentrifugation and circular dichroism show that gloverin exists as a monomeric random coil in water solution, while, according to circular dichroism, an alpha helix structure can be induced by the addition of hexafluoro-isopropanol. The direct measurement of molecular weight by mass spectrometry compared to the mass deduced from the amino acid sequence indicates that the gloverin is not subject to post-translational modifications, e.g. glycosylation.

A protein corresponding to gloverin was isolated from the closely related lepidopteran *Hyalophora cecropia* and the sequence for the 38 N-terminal amino acids was found to be identical. Also the amino acid analysis for the two proteins gave similar results. Comparison of the gloverin sequence with those of other proteins found in the data base disclosed no structural similarity to known proteins. Thus, we conclude that gloverin represents a novel class of antibacterial proteins.

The antibacterial effect of gloverin seems to be directed towards certain gram-negative bacteria. The sensitivity of *E. coli* K-12 increases with decreasing length of the polysaccharide chain of the lipopolysaccharide (LPS). Strain D21f2 used in the experiments is an LPS mutant with an Re-type of LPS, and the most sensitive strain. The parent strain D21(LPS Ra) is about 10 times less sensitive. The fact that gloverin renders these bacteria sensitive to the detergent Triton X-100 and to lysozyme, - compounds that are normally inactive against these cells due to their inability to penetrate the outer membrane - indicates that gloverin has an effect directed against the cell envelope. This effect could be almost completely inhibited by Mg^{2+} that is known to have an important role in stabilisation of the outer membrane of gram-negative bacteria. The observed increase in permeability is accompanied by a decrease in outer membrane proteins, an effect which further indicates that the outer membrane is the target for gloverin.

The observation that the sensitivity of the cell to gloverin increases with decreasing length of the polysaccharide chain of LPS, in combination with the fact that the effect of gloverin on growth is inhibited by pre-incubation with LPS in solution, indicates that binding to LPS is important for the action of gloverin. The polysaccharide chains of LPS may hinder gloverin simply by steric interactions. The shorter the chain, the easier it is for gloverin to get access to the inner parts of the LPS-layer. Possible binding sites for the basic gloverin might be provided by the lipid A part of LPS and/or the phosphate groups present both on lipid A and on the 2-keto-3-deoxyoctonic acid (KDO). The activity of gloverin resembles in many respects (permeability, Omp synthesis, inhibition by free LPS) that of attacin (6,7)

A comparison of gloverin and mammalian BPI, shows that the effect of BPI is also inversely dependant of the length of the LPS polysaccharide chains [14]. Addition of magnesium ions also inhibits the effect of BPI. However, in contrast to gloverin and attacin, BPI does not seem to have the same profound effect on the synthesis of outer membrane proteins [15]. Gloverin retains its antibacterial properties after boiling which shows that the activity is not due to any catalytic effect. This is also true for the attacins, cecropins and BPI.

The novel antibacterial proteins of the invention enable new antimicrobial therapy with new antimicrobial agents, i.e. gloverins. Gloverin can be combined with other conventional antimicrobial agents, such as penicillins, to enhance the antimicrobial effect. Furthermore, they provide useful tools for studies of the regulation of assembly and synthesis of the bacterial outer membrane.

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- 16a -

SEQUENCE LISTING

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 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: CA 2,221,793
 - (B) FILING DATE: 27-JAN-1998
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: SE 9700244-8
 - (B) FILING DATE: 28-JAN-1997
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: SMART & BIGGAR, ,
 - (C) REFERENCE/DOCKET NUMBER: 63786-116
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (613)-232 2486
 - (B) TELEFAX: (613)-232 8440

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 130 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

- 16b -

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp	Val	Thr	Trp	Asp	Lys	Asn	Ile	Gly	Asn	Gly	Lys	Val	Phe	Gly	Thr	1	5	10	15
Leu	Gly	Gln	Asn	Asp	Asp	Gly	Leu	Phe	Gly	Lys	Ala	Gly	Phe	Lys	Gln	20	25	30	
Gln	Phe	Phe	Asn	Asp	Asp	Arg	Gly	Lys	Phe	Glu	Gly	Gln	Ala	Tyr	Gly	35	40	45	
Thr	Arg	Val	Leu	Gly	Pro	Ala	Gly	Gly	Thr	Thr	Asn	Phe	Gly	Gly	Arg	50	55	60	
Leu	Asp	Trp	Ser	Asp	Lys	Asn	Ala	Asn	Ala	Ala	Leu	Asp	Ile	Ser	Lys	65	70	75	80
Gln	Ile	Gly	Gly	Arg	Pro	Asn	Leu	Ser	Ala	Ser	Gly	Ala	Gly	Val	Trp	85	90	95	
Asp	Phe	Asp	Lys	Asn	Thr	Arg	Leu	Ser	Ala	Gly	Gly	Ser	Leu	Ser	Thr	100	105	110	
Met	Gly	Arg	Gly	Lys	Pro	Asp	Val	Gly	Val	His	Ala	Gln	Phe	Gln	His	115	120	125	
Asp	Phe															130			

63786-116

17

CLAIMS:

1. An antibacterial protein against gram negative bacteria, said protein having an approximate molecular weight of 14 kD and an approximate isoelectric point of 9.
- 5 2. An antibacterial protein according to claim 1, derived from *Lepidoptera*.
3. An antibacterial protein according to claim 2, derived from *Hyalophora* moths, wherein the molecular weight is approximately 13.8 kD and the pI is 8.5.
- 10 4. An antibacterial protein according to claim 3, said protein having the amino acid sequence shown in Fig. 2 or an amino acid sequence substantially homologous therewith.
5. An antibacterial protein according to claim 4,
15 said protein being encoded by a DNA sequence derived from the amino acid sequence shown in Fig. 2 or from an amino acid sequence substantially homologous therewith.
6. An antibacterial protein according to claim 1, said protein produced by genetic engineering or chemical
20 synthesis.
7. A pharmaceutical composition, comprising: an antibacterial protein according to any one of claims 1 - 6 or any antibacterially active fragment thereof; and a pharmaceutically acceptable diluent or carrier.
- 25 8. A pharmaceutical composition according to claim 7, further comprising at least one conventional antibacterial agent.
9. A pharmaceutical composition according to claim 8, wherein said conventional antibacterial agents are selected

63786-116

18

from the group consisting of antibiotics and cell-degrading agents.

10. An antibacterial protein according to claim 1 or pharmaceutically active fragments thereof for use as a
5 medicament.

11. Use of an antibacterial protein according to claim 1 to treat infections caused by gram-negative bacteria.

12. Use of an antibacterial protein according to claim 1 in combination with at least one conventional
10 antibacterial agent to treat bacterial infection.

13. Use of an antibacterial protein according to claim 1 in the preparation of a medicament for treating bacterial infection.

14. The use according to claim 13, further comprising
15 use of at least one conventional antibacterial agent.

15. A pharmaceutical composition according to claim 9, wherein said cell-degrading agents are selected from the group consisting of enzymes and detergents.

SMART & BIGGAR

OTTAWA, CANADA

PATENT AGENTS

1/8



Agents
1997

FIG. 1

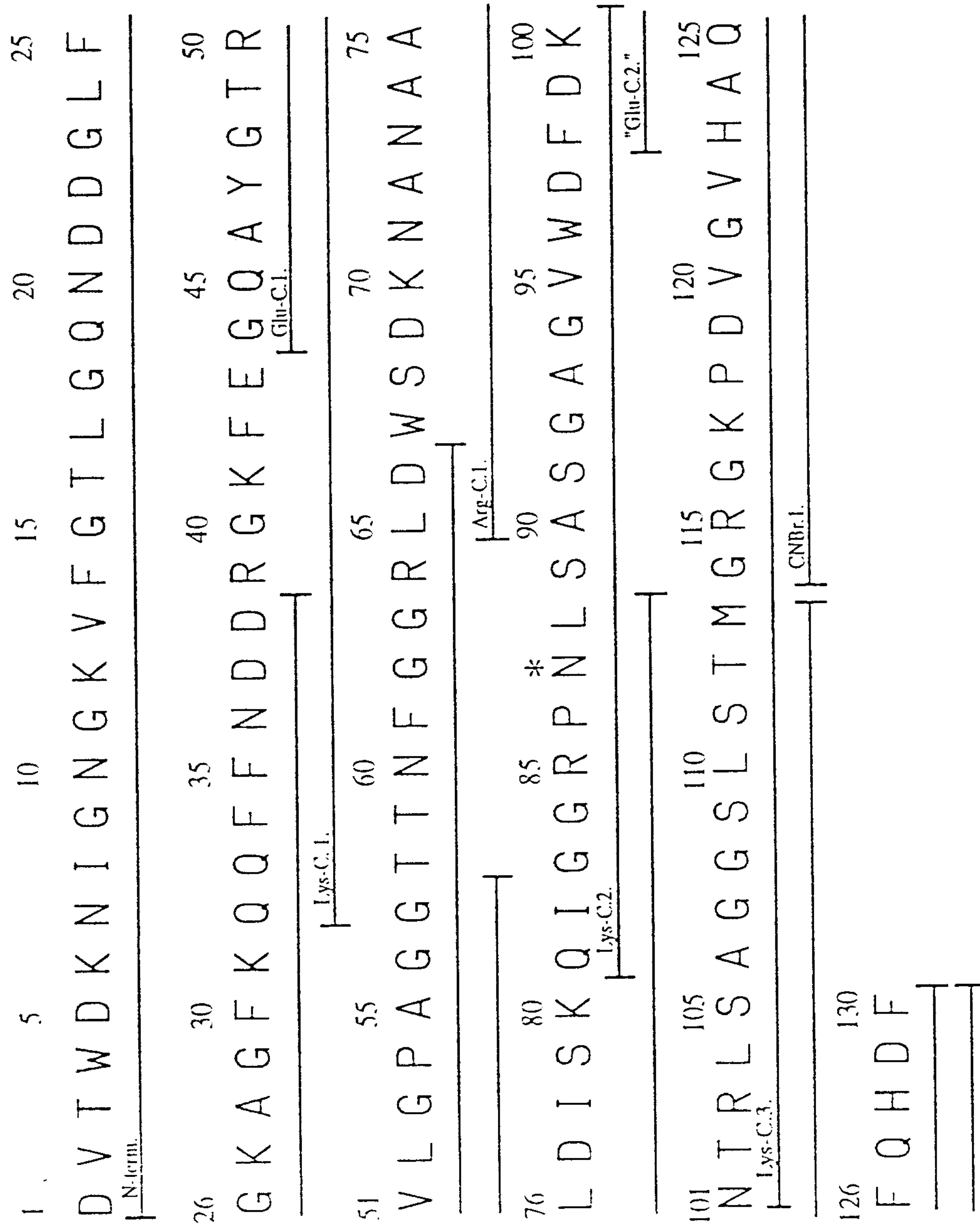


FIG. 2

1000
1000

3/8

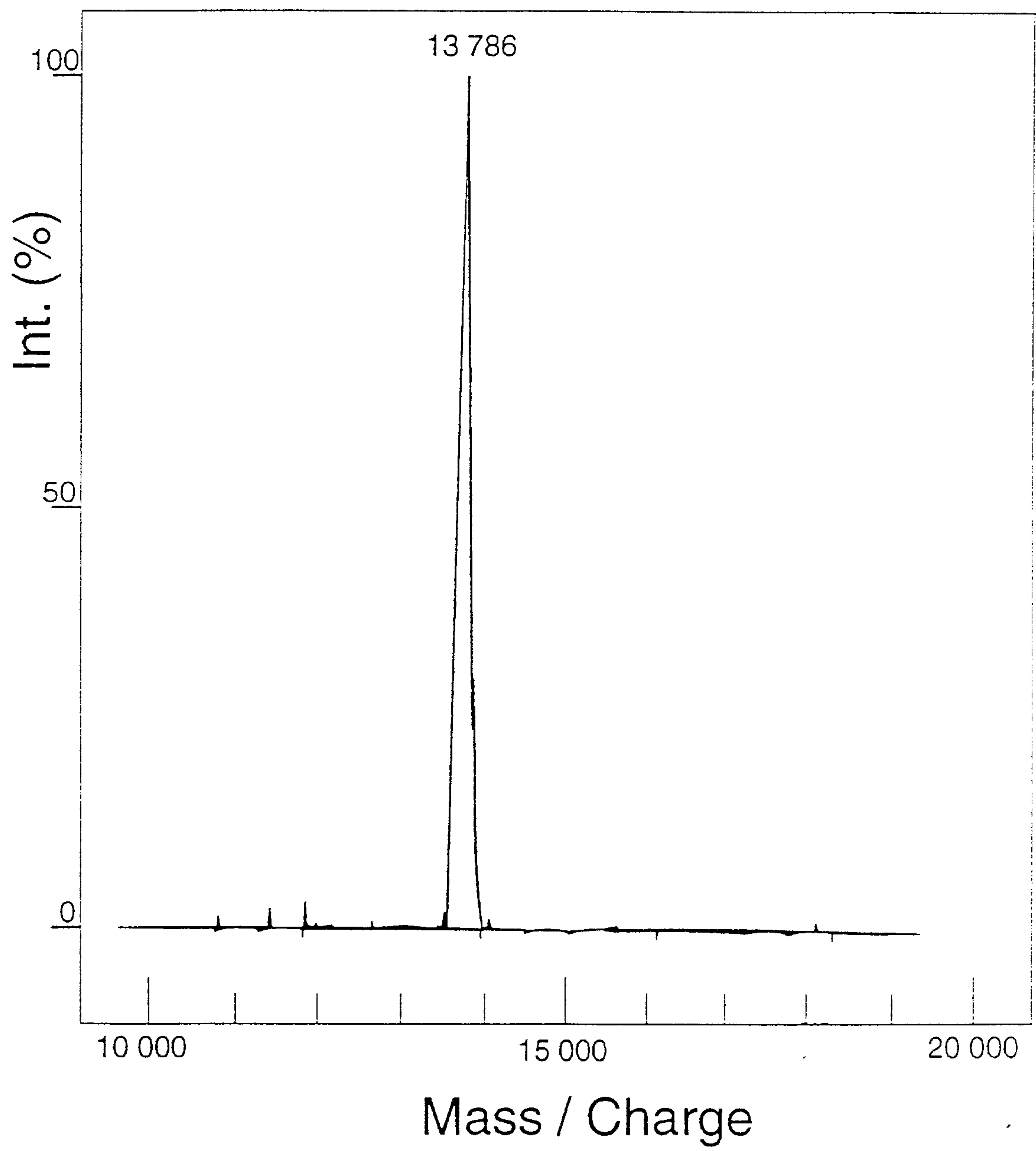


FIG. 3

FIG. 3
Mass spectrum of the polymer

4/8

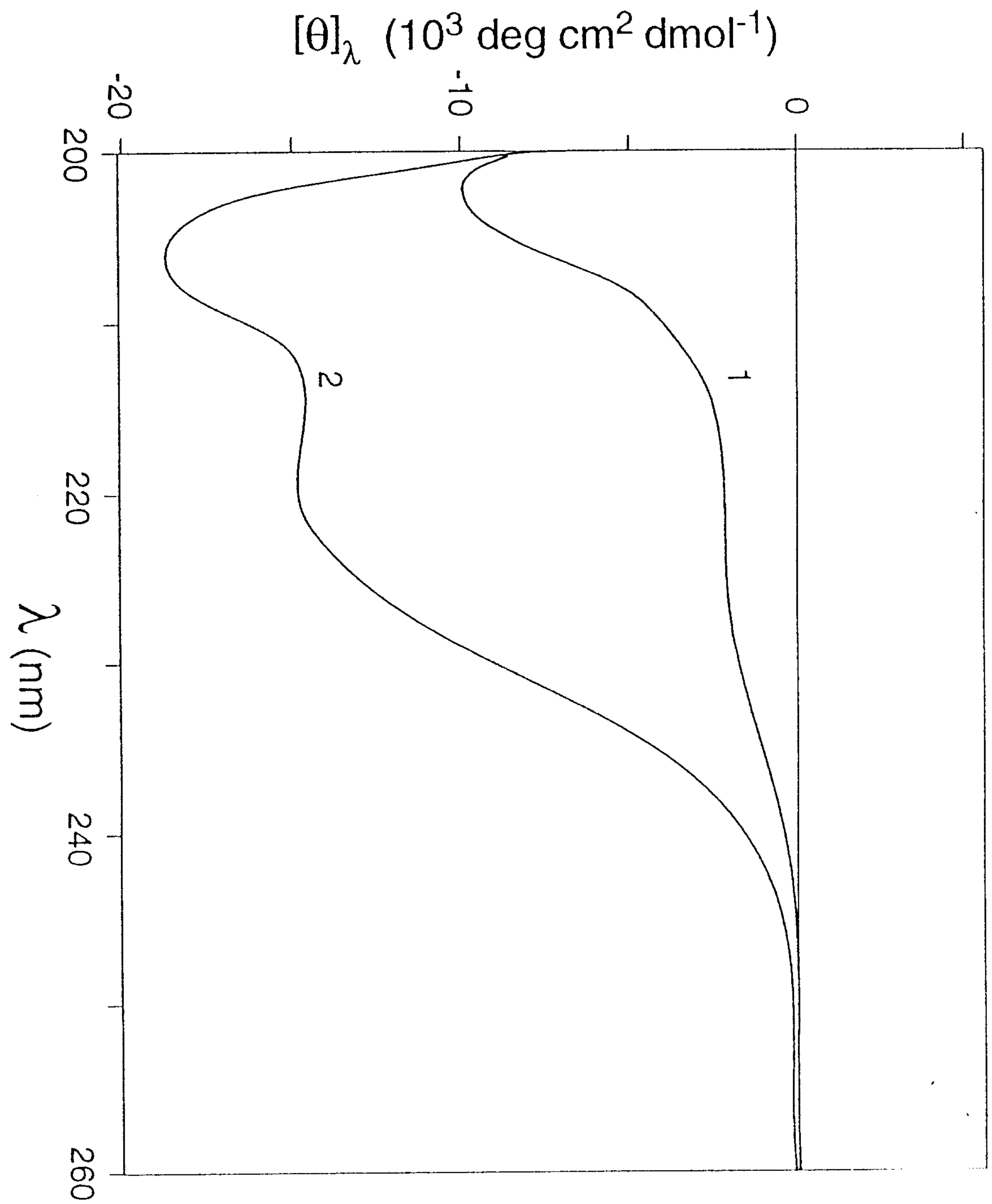


FIG. 4

5/8

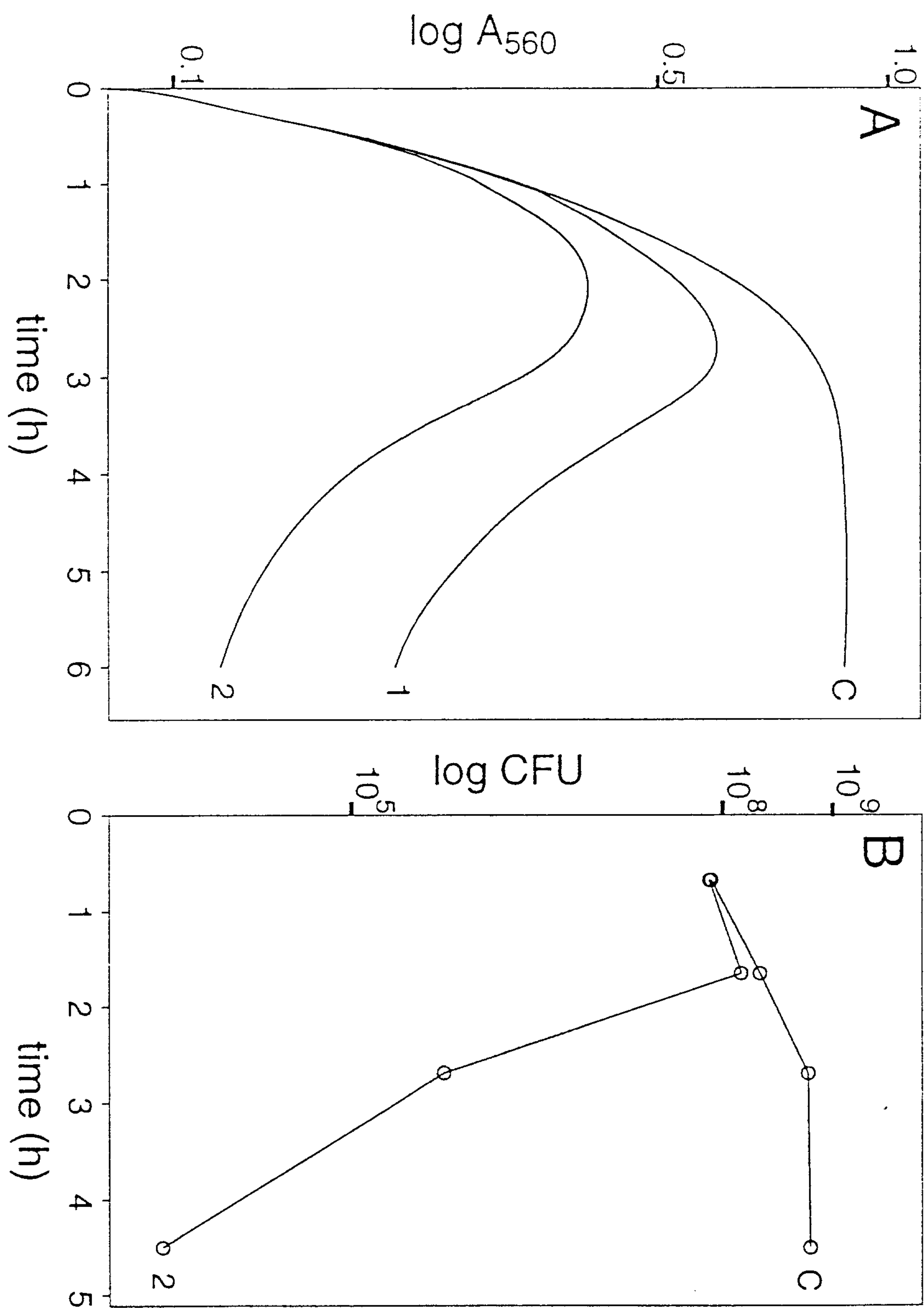


FIG. 5

6/8

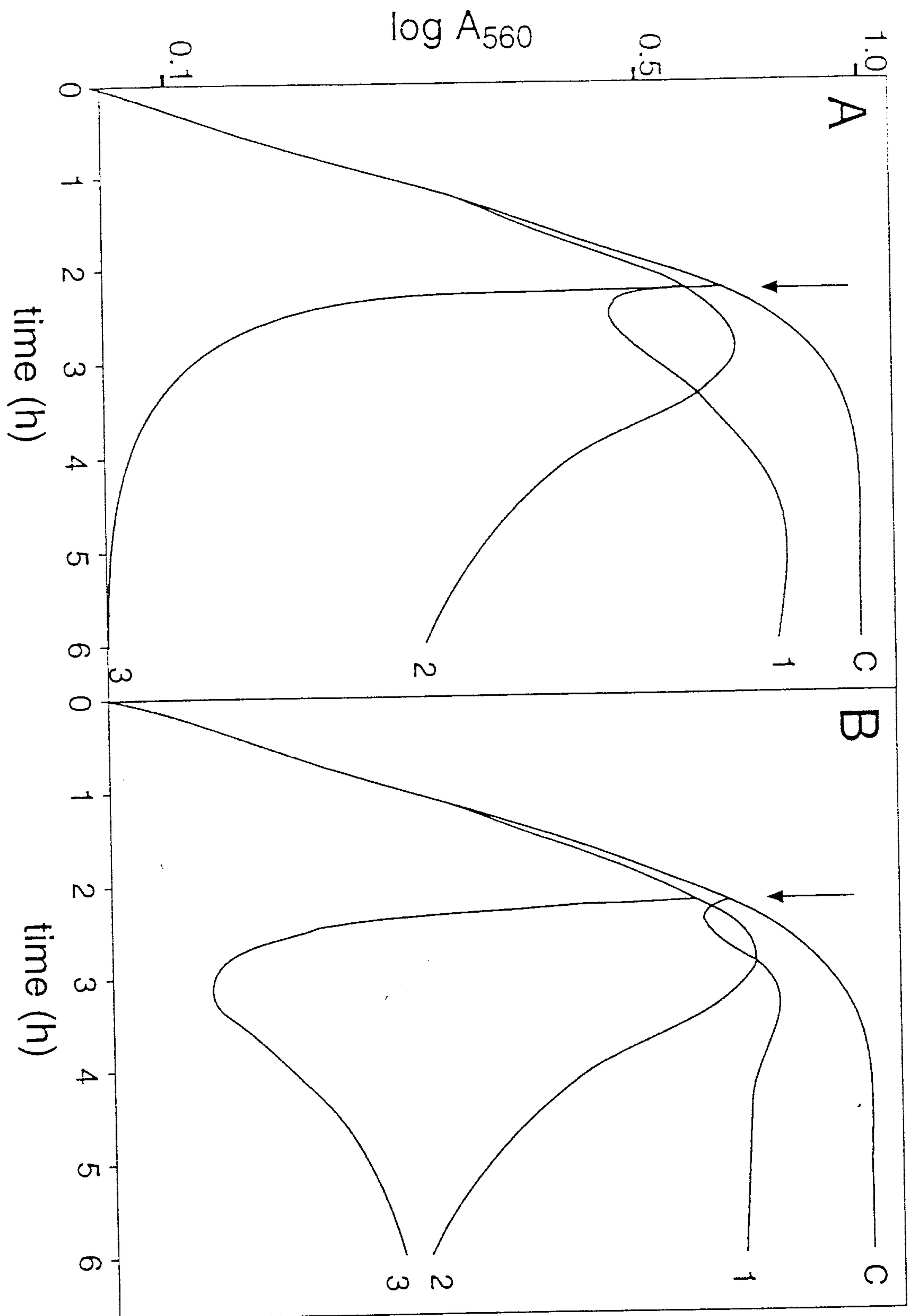


FIG. 6

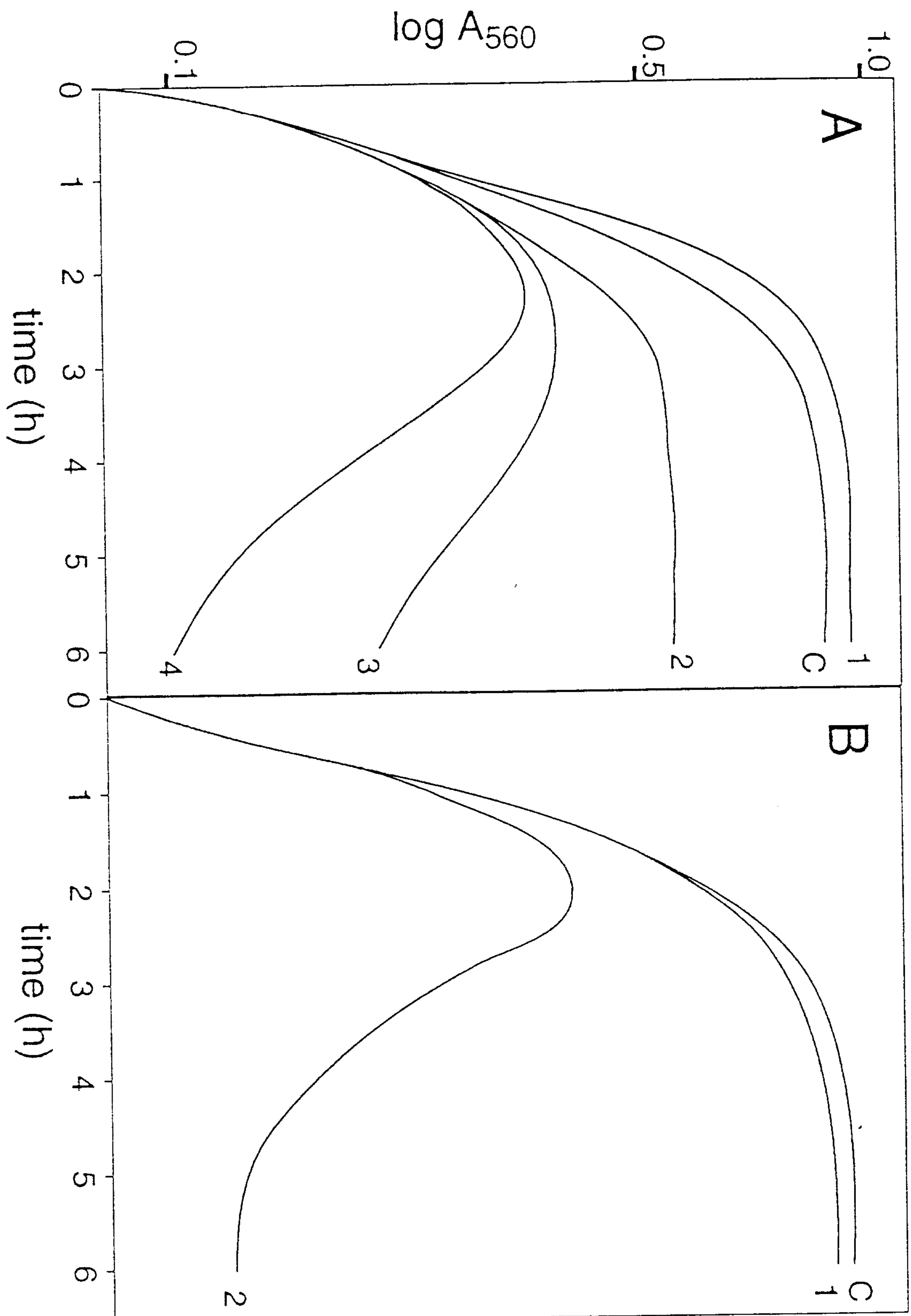


FIG. 7

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 100-443884

8/8

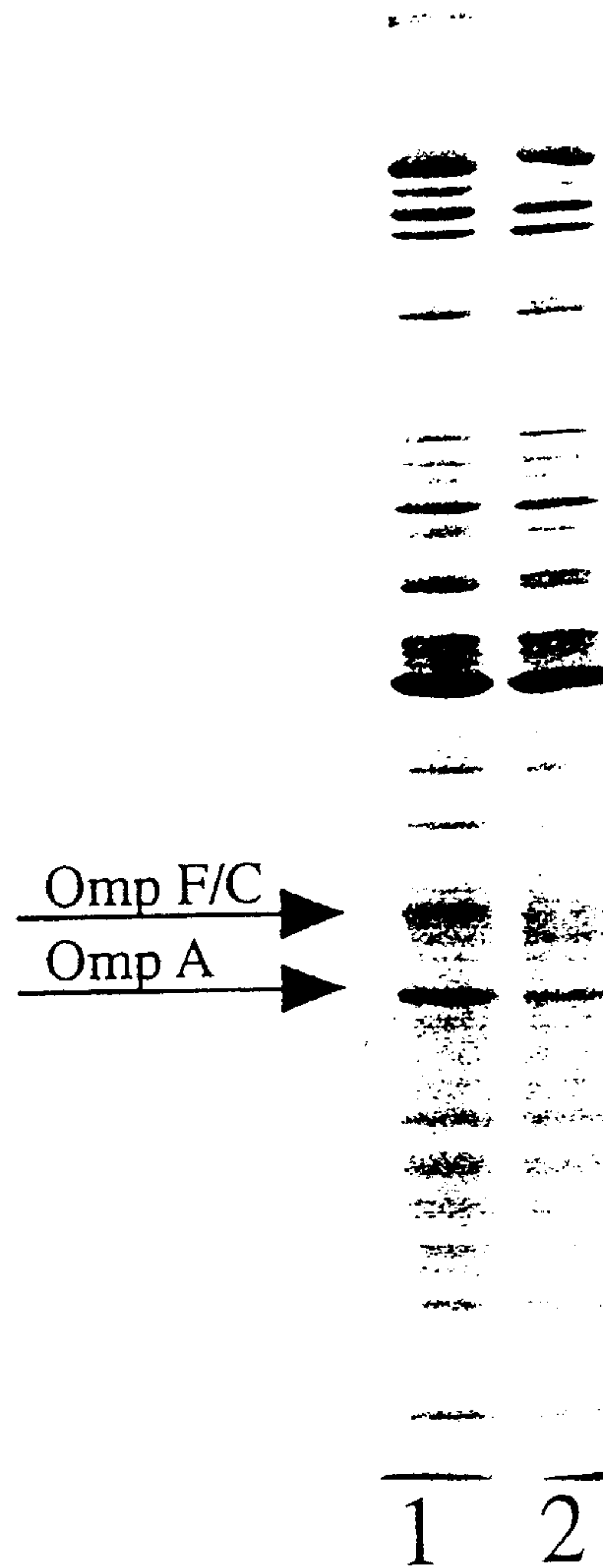


FIG. 8

J. Biol. Chem. 273:10113-10118 (1998)

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1 5 10 15 20 25
D V T W D K N I G N G K V F G T L G Q N D D G L F
| N-term.

26 30 35 40 45 50
G K A G F K Q Q F F N D D R G K F E G Q A Y G T R
| Lys-C.1. | Glu-C.1.

51 55 60 65 70 75
V L G P A G G T T N F G G R L D W S D K N A N A A
| Arg-C.1.

76 80 85 * 90 95 100
L D I S K Q I G G R P N L S A S G A G V W D F D K
| Lys-C.2. | "Glu-C.2."

101 105 110 115 120 125
N T R L S A G G S L S T M G R G K P D V G V H A Q
| Lys-C.3. | CNBr.1.

126 130
F Q H D F
|
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