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(54) Titre : METHODE DE PURIFICATION DE POLYPEPTIDES
(54) Title: PROCESS FOR PURIFICATION OF POLYPEPTIDE

(57) **Abrégé/Abstract:**

An improved process for purifying a polypeptide using a packing material for reversed phase high performance liquid chromatography is provided. A process for purifying a polypeptide characterized in that an aqueous solution containing polypeptide obtained by pre-treating a polypeptide produced by a wide variety of cells to a predetermined state is adjusted to a specific pH value, to remove impurities, and is then treated with a packing material for reversed phase high performance liquid chromatography.



ABSTRACT

An improved process for purifying a polypeptide using a packing material for reversed phase high performance liquid chromatography is provided. A process for purifying a polypeptide characterized in that an aqueous solution containing polypeptide obtained by pre-treating a polypeptide produced by a wide variety of cells to a predetermined state is adjusted to a specific pH value, to remove impurities, and is then treated with a packing material for reversed phase high performance liquid chromatography.

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DESCRIPTIONPROCESS FOR PURIFICATION OF POLYPEPTIDE

[Technical Field]

The present invention relates to an improved process for purifying a polypeptide, more specifically, to a purification process carried out by
5 subjecting an objective substance containing a polypeptide to a pretreatment, and then treating the resulting crude polypeptide aqueous solution with a packing material for reversed phase high performance liquid chromatography.

10 [Background Art]

A very complicated procedure is required to purify polypeptides produced by microorganisms, animal cells, and plant cells, while maintaining their physiological activities to high degree.
15 Consequently, the present procedures require some improvement. For example, the purification of a human growth hormone releasing factor produced by transformed microorganisms involves a ten stage procedure, resulting in a large amount of production
20 but at a yield too low for carrying out a bioassay (Vincent Geli et al., Gene, 80, 129-136 (1989)). For the purification of human calcitonin, it has been reported that an eight stage purification is carried out, using 6 types of columns, to isolate human
25 calcitonin (J. P. Gilligan et al., Biochromatography, 2 (1), 20-27 (1987)).

These purification steps, however, are very complicated, and thus it may be considered that they lead to the decomposition of polypeptides, and to the
30 disappearance of physiological activities of polypeptides during the purification.

The object of the present invention is, therefore, to provide a process which can isolate

polypeptides in a stable form and isolate and purify polypeptides at a high yield by carrying out a simple procedure, in order to thus solve these problems.

[Disclosure of the Invention]

5 Over the past several years, various physiologically active polypeptides, represented by the human growth hormone and human calcitonin, have been increasingly produced with the aid of various genetically engineered cell lines. Of these, in
10 addition to naturally found types of physiologically active polypeptides per se, there are many polypeptides produced as fused polypeptides (also referred to as "chimera proteins") to which other protein moieties are fused. Although these can be
15 purified by using a conventional separation/purification process, there has been a particular desire for the development of a process for efficiently recovering objective physiologically active polypeptides without any deactivation after
20 cleaving fused polypeptides into physiologically active moieties and other protein moieties fused thereto. The present inventors found that, when the cleaved substances of the above-mentioned fused polypeptides are treated under a specific pH level,
25 and the treated liquid thus obtained is treated with a packing material for reversed phase high performance liquid chromatography, objective physiologically active polypeptides can be efficiently obtained, and that this process also can be advantageously used for
30 purifying samples containing physiologically active polypeptides per se, to thereby accomplish the present invention.

The above-mentioned object can be achieved by providing a process for purifying a polypeptide of the
35 present invention, which process comprises the following steps of

(a) regulating the pH range of an aqueous solution containing a crude polypeptide to 1-4 to cause impurities to precipitate, followed by removing these impurities, and

5 (b) adsorbing the supernatant obtained in the above-mentioned stage (a) on a packing material for reversed phase high performance liquid chromatography, followed by eluting a desired polypeptide.

[Brief Description of the Drawings]

10 Figs. 1 (a) - (e) show HPLC elution patterns of human calcitonin precursor solutions purified according to the process of the present invention according to the sequence of steps; Fig. 2 shows an HPLC elution pattern of the specimen of Fig. 1 (e) after having
15 been freeze-dried; Fig. 3 shows an HPLC elution pattern of the highly purified human calcitonin precursor obtained by dispensing the solution of Fig. 2; Fig. 4 shows an elution pattern of a human calcitonin fused polypeptide using an ion-exchange
20 column chromatography; Fig. 5 shows an HPLC elution pattern of an eluate purified according to the process of the present invention; and Fig. 6 shows an HPLC elution pattern of melanocyte-stimulating hormone eluate purified according to the process of the
25 present invention.

[Best Mode of Carrying Out the Invention]

The polypeptides to be purified according to the present invention may originate from microorganisms, animal cells and plant cells, or from the cells
30 which have been genetically engineered to produce desired polypeptides. Consequently, the purification process of the present invention is aimed at treated substances (e.g. homogenates) and/or cultures of the above-mentioned cells.

35 Before these treated substances and/or cultures are subjected to the process of the present invention, cell homogenate substances or the cells themselves

are removed, objective physiologically active polypeptides are solubilized in an aqueous medium, and optionally are concentrated, to be purified in a separation/purification process known per se. Where
5 the physiologically active polypeptides are obtained from the above-mentioned origins in the fused polypeptide form, they are purified according to the process of the present invention, after being purified to a considerably high degree in the fused polypeptide
10 form, and then are cleaved into the physiologically active moieties and other protein moieties. Therefore, the term "aqueous solution containing crude polypeptides" used in the present invention includes a wide variety of treated liquids coming from the above-
15 mentioned origins, which can be applied in any purification stage as long as the effect of the present invention is exhibited. One kind of solution which can be advantageously treated according to the process of the present invention includes, but is not
20 limited to, a reaction solution after fused polypeptides are cleaved into the physiologically active moieties and other protein moieties.

The treated substances and/or cultures of the above-mentioned cells can be prepared by a process for
25 producing polypeptides known per se.

For example, the outline for the production of polypeptide using an expression vector is as follows:

As hosts which express genes coding for polypeptides, microorganisms such as E. coli, Bacillus subtilis, yeasts; animal cells such as those
30 originating from insects, mammals, and the amphiba; and plant cells can be mentioned. As the expression vector, any plasmid can be used as long as it can effectively express a gene encoding a desired
35 polypeptide in the cells. For example, it can be suitably selected from plasmids described in the following literature:

Vector DNA, the 1st press (1986), edited by Yoshiyuki Sakaki., Kodansha; Zoku Seikagaku Jikken Koza I, Idenshi Kenkyuhou II (How to Research Gene II), - Kumikae DNA Gijutsu (DNA Recombination Technique)-, Chapter 7, Kumikaetai no Hatsugen (Expression of Recombinants), edited by Society of Biochemical Society of Japan, Tokyo Kagaku Dojin; Recombinant DNA, Part D, Section II, Vectors for Expression of Cloned Gene, (1987), edited by Ray Wu and Lawrence Grossman., Academic Press, INC: Molecular Cloning, A Laboratory Manual 2nd Ed, Book 3, (1989), edited by J. Sambrook, E. P. P. Pritsch and T. Maniatis, Cold Spring Harbor Laboratory Press; etc.

For example, in the case of E.coli, pMb, pBR, and pUC type vectors, for yeasts, YIp, YRp, or YEp type vectors, and for Bacillus subtilis, pUB, pBC, or pBD types can be used. For animal cells, SV 40, BKV, or BPV types can be used. For plant cells, the same vectors as those in the case of E. coli, with the exception that the promoters are changed to those which work in the plants, can be used. Examples of

promoters working in the plants include promoters for chlorophyll a-b binding proteins, cauliflower mosaic virus 35S, and the like.

The recombination of these vectors, and the transformation and transduction of the host cell with the recombinant plasmids can be carried out by procedures known per se described in the above-mentioned literature, etc., respectively. The transformed cells thus obtained can be cultivated in a medium under the culture conditions usually used for growing the cell to be treated.

Where the polypeptides and/or fused polypeptides from such cultivated substances are secreted extracellularly, the cells are removed, and where they are accumulated in the cell, after the culture is

removed, the polypeptides and/or fused polypeptides are collected by cell homogenization, etc.

Although not intended to be restricted, the polypeptides at which the purification according to the present invention is aimed are those in which two or more amino acids are peptide-bonded. Also the term "polypeptides" used herein is intended to include modified polypeptides, such as the polypeptides in which saccharide or phosphoric acid is bonded to their amino acids and polypeptides whose N-terminal end is amidated, etc. Such polypeptides possess a molecular weight of not less than 15,000, and include, for example, hormones and growth factors such as insulin, growth hormone release factor (GRF), epidermal growth factor-(EGF), atrial natriuretic peptide (ANP), thymosin α_1 , thymosin β_4 , thymopoietin, transforming growth factor (TGF- α), adrenocorticotrophic hormone (ACTH), calcitonin gene-related peptide (CGRP), and cartilage factor (CDF); and cytokinins such as interleukin-2 and interleukin-3. Polypeptides which can be preferably applied to the process of the present invention other than these polypeptides include the polypeptides listed below.

As an explanation of the polypeptides, when amino acids and other things are displayed as abbreviations, they are displayed according to IUPAC rules or by symbols usual in this field. Some examples thereof are listed below.

	Ser: L-serine	Leu: L-leucine
	Arg: L-arginine	Cys: L-cysteine
30	Gln: L-glutamine	Lys: L-Lysine
	Ile: L-isoleucine	
	Pro: L-proline	Val: L-valine
	His: L-histidine	Met: L-methionine
35	Ala: L-alanine	Gly: Glycine
	Phe: L-phenylalanine	
	Asp: L-aspartic acid	

Asn: L-asparagine
Glu: L-glutamic acid
Trp: L-tryptophan
Thr: L-threonine
5 Tyr: L-tyrosine
x: any one of the above-mentioned amino acids
hCT: human calcitonin
CT: calcitonin
HPLC: high performance liquid chromatography
10 (1) Angiotensin II which can be used as an
angiotonic or a hypertensioning agent
(originating from equine)
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
(L. T. Skeggs et al., J. Exptl. Med, 106,
15 439, 1957)
(2) Angiotensin II antagonist known as a
hypotensor
Ser-Arg-Val-Tyr-Val-His-Pro-Ala
(3) Angiotensin III
20 Arg-Val-Tyr-Ile-His-Pro-Phe
(Campbell. W. B. et al., Science, 184, 994, 1974)
(4) C-Terminal glycine adduct of calcitocin
known as know as a hyperkalemia treating
agent (precursor for C-terminal amidation)
25 (Human)
Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-
Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-
Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-
Gly-Val-Gly-Ala-Pro-Gly
30 (Swine)
Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-
Ser-Ala-Tyr-Trp-Arg-Asn-Leu-Asn-Asn-
Phe-His-Arg-Phe-Ser-Gly-Met-Gly-Phe-
Gly-Pro-Glu-Thr-Pro-Gly
35 (Bovine)
Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-
Ser-Ala-Tyr-Trp-Lys-Asp-Leu-Asn-Asn-

Tyr-His-Arg-Phe-Ser-Gly-Met-Gly-Phe-
Gly-Pro-Glu-Thr-Pro-Gly

(Salmon)

5 Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-
Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-
Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-
Gly-Ser-Gly-Thr-Pro-Gly

(Rabbit)

10 Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-
Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-
Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asp-Val-
Gly-Ala-Gly-Thr-Pro-Gly

(Avian)

15 Cys-Ala-Ser-Leu-Ser-Thr-Cys-Val-Leu-
Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-
Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asp-Val-
Gly-Ala-Gly-Thr-Pro-Gly

(Lasmoles. F., et al., FEBS lett. 180, 113, 1985)

20 (5) Melanocyte-stimulating hormone having a
melanocyte-stimulating effect, α -MSH
Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-
Gly-Lys-Pro-Val

(Harris, J. I. et al., Nature, 179, 1346, 1957)

25 (6) Melanocyte-stimulating hormone, β -MSH (Squalidae)
Asp-Gly-Asp-Asp-Tyr-Lys-Phe-Gly-His-
Phe-Arg-Trp-Ser-Val-Pro-Leu
(Bennet, H. P. J. et al., Biochem. J., 141,
439, 1974)

(7) Trypsin inhibitor

30 (Human)

35 Asp-Ser-Leu-Gly-Arg-Glu-Ala-Lys-Cys-
Tyr-Asn-Glu-Leu-Asn-Gly-Cys-Thr-Lys-
Ile-Tyr-Asn-Pro-Val-Cys-Gly-Thr-Asp-
Gly-Asp-Thr-Tyr-Pro-Asn-Gly-Cys-Val-
Leu-Cys-Phe-Glu-Asn-Arg-Lys-Arg-Gln-
Thr-Ser-Ile-Leu-Ile-Gln-Lys-Ser-Gly-
Pro-Cys

(Bartelt. D.C. et al., Arch. Biochem. Biophys.,
179, 189, 1977)

(Bovine)

5 Asn-Ile-Leu-Gly-Arg-Glu-Ala-Lys-Cys-
Thr-Asn-Glu-Val-Asn-Gly-Cys-Pro-Arg-
Ile-Tyr-Asn-Pro-Val-Cys-Gly-Thr-Asp-
Gly-Val-Thr-Tyr-Ser-Asn-Glu-Cys-Leu-
Leu-Cys-Met-Glu-Asn-Lys-Glu-Arg-Gln-
Thr-Pro-Val-Leu-Ile-Gln-Lys-Ser-Gly-
10 Pro-Cys

(Greene, L. J. et al., J. Biol. Chem. 244, 2646,
1969)

(8) Accessory thyroid hormone having calcium
release effect

15 (Swine)

Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-
Asn-Leu-Gly-Lys-His-Leu-Ser-Ser-Leu-
Glu-Arg-Val-Gln-Trp-Leu-Arg-Lys-Lys-
Leu-Gln-Asp-Val-His-Asn-Phe-Val-Ala-
20 Leu-Gly-Ala-Ser-Ile-Val-His-Arg-Asp-
Gly-Gly-Ser-Gln-Arg-Pro-Arg-Lys-Lys-
Glu-Asp-Asn-Val-Leu-Val-Glu-Ser-His-
Gln-Lys-Ser-Leu-Gly-Glu-Ala-Asp-Lys-
Ala-Ala-Val-Asp-Val-Leu-Ile-Lys-Ala-
25 Lys-Pro-Gln

(Brewer, H. B., et al., Amer. J. Med., 56, 759,
1974)

(9) Avoidance inducing hypophysis peptide
(Swine)

30 Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys
(Lande, S., et al., J. Biol. Chem., 246,
2058, 1971)

(10) Proinsulin C peptide
(Bovine)

35 Glu-Val-Glu-Gly-Pro-Gln-Val-Gly-Ala-
Leu-Glu-Leu-Ala-Gly-Gly-Pro-Gly-Ala-
Gly-Gly-Leu-Glu-Gly-Pro-Pro-Gln

(Salokangas, A. et al., Eur. J. Biochem., 20, 813, 1971)

(11) Insulin-like growth factor I known as a cell growth promoting factor

5

Gly-Pro-Glu-Thr-Leu-Cys-Gly-Ala-Glu-
Leu-Val-Asp-Ala-Leu-Gln-Phe-Val-Cys-
Gly-Asp-Arg-Gly-Phe-Tyr-Phe-Asn-Lys-
Pro-Thr-Gly-Tyr-Gly-Ser-Ser-Ser-Arg-
Arg-Ala-Pro-Gln-Thr-Gly-Ile-Val-Asp-
10 Glu-Cys-Cys-Phe-Arg-Ser-Cys-Asp-Leu-
Arg-Arg-Leu-Glu-Met-Tyr-Cys-Ala-Pro-
Leu-Lys-Pro-Ala-Lys-Ser-Ala

(Rinderknecht, E. et al., Proc. Natl. Acad. Sci. USA, 73, 4379, 1976)

15

(12) Pancreatic polypeptide

(Avian)

Gly-Pro-Ser-Gln-Pro-Thr-Tyr-Pro-Gly-
Asp-Asp-Ala-Pro-Val-Glu-Asp-Leu-Ile-
Arg-Phe-Tyr-Asp-Asn-Leu-Gln-Gln-Tyr-
20 Leu-Asn-Val-Val-Thr-Arg-His-Arg-Tyr

(Kimmel, J. R. et al., J. Biol. Chem., 250, 9369, 1978)

25

--(13) Peptides bound a glycyl group to calctonin gene-related peptides at the C-terminal amino acid residue (precursors for C-terminal amidation)

(Human α type)

30

Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-
Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys
Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-
Phe-Gly

35

(Morris et al., Nature, 308, 746 (1984))

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(Human β type)

Ala-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-
Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Met-Val-Lys-
Ser-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-
Phe-Gly

(Steenberg et al., FEBS Lett, 183,403 (1985))

(Rat α type)

Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-
Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-
Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-
Phe-Gly

(Amara et al., Nature, 298,240 (1982))

(Rat β type)

Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-
Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-
Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-
Phe-Gly

(Amara et al., Science, 229,1094 (1985))

(14) Hormone having angiotonic and hyperphagia
effect (Neuro peptide, NPY)

Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Met-
Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-
Asn-Leu-Ile-Tyr-Arg-Gln-Arg-Tyr

(Tatemoto et al., Proc. Natl. Acad. Sci.
USA., 79,5485 (1982))

(15) Growth hormone-releasing factor (GRF)

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-
Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-
Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-
Gln-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-
Arg-Leu

(Mac Gillivray et al., Proc. Natl. Acad. Sci.
USA, 79,2504 (1982))

(16) Secretion

His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Ser-Arg-Leu-
Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-
Gln-Gly-Leu-Val

(Mutt et al., Biochem. Biophys. Res. Commun., 9,275
(1962))

(17) Hormone having hypotensive effect (VIP)

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-
Try-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-
Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn

(Said et al., Eur. J. Biochem., 28,199 (1972))

(18) Hormone PHI having angiectatic and insulin-secretomotory effect (peptide HI)

His-Ala-Asp-Gly-Val-Phe-Thr-Ser-Asp-Phe-Ser-
Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys-Lys-
Thr-Leu-Glu-Ser-Leu-Ile

(Tatemoto et al., Proc. Natl. Acad. Sci.
USA, 75,4115 (1978))

(19) Gastrin-releasing peptide (GRP)

Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-
Thr-Lys-Met-Thr-Pro-Arg-Gly-Asn-His-Trp-Ala-
Val-Gly-His-Leu-Met

(McDonald et al., Biochem. Biophys. Res. Commun.,
90,227 (1979))

(20) Cholecystokinin (CCK)

Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-
Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-
Arg-Ile-Ser-Asp-Arg-Asp-Trp(SO₃⁻)-Met-Gly-
Trp-Met-Asp-Phe-Gly-Arg-Arg-Ser-Ala-Glu

(Mutt et al., Biochem. J., 125,57, (1971))

(21) Hormone PYY suppressing pancreatic juice
secretion

Tyr-Pro-Ala-Lys-Pro-Glu-Ala-Pro-Gly-Glu-Asp-
Ala-Ser-Pro-Glu-Glu-Leu-Ser-Arg-Trgr-Ala-Ser-
Leu-Arg-His-Tyr-Leu-Asn-Leu-Val-Thr-Arg-Gln-
Arg-Tyr

(Tatemoto et al., Nature, 285,417 (1980))

(22) Gastric motor activity-stimulating hormone
(motilin)

Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-
Arg-Met-Gln-Glu-Lys-Glu-Arg. Asn-Lys-Gly-Gln

(Brown; Can. J. Physiol. Pharmacol., 49,399,
(1971))--

Consequently, where genes which code for the above-mentioned physiologically active polypeptides are expressed in an adequate host cell, the fused polypeptides of the present invention are genetic products in which genes, for example, which code for proteins (if necessary, including adequate cleavable portions) making them easily detectable and the above-mentioned genes are artificially ligated. These proteins include β -galactosidase, chloramphenicol acetyltransferase, and the like.

Utilizing the aqueous solution of crude polypeptides prepared as described above, the process of the present invention is preferably carried out while monitoring the objective polypeptides by using the RIA method or HPLC method. Where the fused

polypeptides are obtained as precursors of the objective polypeptides, it is necessary to cleave the objective polypeptide moieties and other protein moieties fused thereto as described above, to prepare an aqueous solution containing the crude polypeptides of the present invention. Techniques for this cleavage may be selected according to the type of polypeptide, but generally processes of treating with CNBr, trypsin, collagenase, etc., are applicable. In this case, to inhibit non-specific peptidase activity, it is preferred to add an adequate amount of protease inhibitors, such as N-ethylmaleimide (NEM), dithiothreitol (DTT), 2-mercaptoethanol (2-ME), ethylenediamine tetraacetic acid (EDTA), or phenylmethanesulfonylfluoride (PMSF).

The reaction product, i.e., an aqueous solution containing crude polypeptides of the present invention, is then purified in a purification stage. For example, in a reaction solution of crude polypeptides obtained by cleaving with collagenase, formic acid, acetic acid, hydrochloric acid, or an aqueous solution thereof is added to adjust the pH to 1-4, preferably about pH 2. If the pH level exceeds 4, immanent protease, or non-specific protease, which possibly co-exists in the collagenase, adversely affects the stability of the desired physiologically active polypeptides, and the impurities to be removed may not be sufficiently modified and precipitated. If the pH level is less than 1, a precipitation of the objective polypeptides may occur, which would result in a worsened recovery rate. As the acid, formic acid is most preferable. The impurities thus precipitated are filtered or subjected to centrifugal separation. For example, after the solution is left to stand, the impurities precipitated by centrifugal separation are removed, thereby obtaining a supernatant having the desired polypeptides dissolved therein. With regard

to the revolution number of the centrifugal separation at this time, this step is preferably carried out at 1000 to 100000, especially 5000 to 30000.

5 If the separation is carried out at a revolution number of less than 1000, removal of the impurities may be insufficient. Even if the revolution number is more than 100000, no significant effect can be obtained.

10 The above-mentioned stage is preferably carried out at a temperature equal to or less than normal room temperature, particularly at 1 to 15°C. If the temperature is less than 0°C, the solution is frozen, so that the stability of the polypeptides is lowered when

15 they are melted again. On the other hand, if the temperature exceeds 15°C, the stabilities of the desired polypeptides may also be lowered. The period for treating with the acid is from several minutes to several hours, and usually a sufficient effect can be obtained at about 30 minutes. If the treatment period is less than several minutes, the impurities may be

20 insufficiently removed. A treatment period over several hours gives no significant added effect.

The acid solution having the objective polypeptides dissolved therein obtained in the former stage is then adsorbed on a packing material for

25 reversed phase high performance liquid chromatography. Any adsorption method able to bring a carrier into contact with the polypeptide in the solution can be applied as a means for adsorption. For example, an

30 adsorption method in which an adequate amount of carrier is incorporated in a solution having a desired polypeptide dissolved therein, the contact being promoted by stirring or shaking to be adsorbed, an

35 adsorption method in which a carrier is packed in a tube made of a suitable material, the polypeptide solution being passed through the tube to be adsorbed, an adsorption method in which a carrier is set as a

filter bed, the peptide solution being passed and adsorbed thereon by pouring it thereon, etc., may be mentioned, but the method is not limited thereto as long as the peptide is brought into contact with a carrier, to thereby adsorb the peptide on the carrier.

As the packing material for reversed phase high performance liquid chromatography, a material in which cyanol groups having substituents of various carbon numbers being bonded on its surface can be used.

Examples of commercially available products include CAPCELL PAK^{*}C₁₈ SG 300, CAPCELL PAK C₈ SG 300, CAPCELL PAK C₁₈ AG 120, and CAPCELL PAK C₈ AG 120 (all produced by Shiseido), Superpacks ferisoap ODS2^{*} (produced by Pharmacia), TSK^{*} gel ODS-80TM, TSK gel ODS-120A, and TSK gel ODS-120T (all produced by Tosoh), Hipore RP-304 C₄^{*} and Hipore RP-318 C₁₈^{*} (both produced by Bio-Rad Laboratory), and the like.

The elution of the polypeptide adsorbed can be carried out after washing with an aqueous 0.1% trifluoroacetic acid solution (for amino acid analysis), by changing the polarity of the adsorbed polypeptide with a polar solvent such as acetonitrile, methanol, or butanol.

EXAMPLE

The present invention will now be described in detail with reference to the working examples, but the present invention is not to be limited thereto.

Example: Purification of Human Calcitonin Precursor produced by transforming E. coli

Preparation of fused Polypeptide (Referential Example)

To obtain a human calcitonin precursor (which was then amidated at the C terminal to be human calcitonin), a gene which codes for human calcitonin-collagenase cleavage portion peptide- β -galactosidase fused polypeptide was prepared and the gene was

incorporated in E. coli to be expressed. The transformed microorganism was cultivated in the manner described below.

To be specific, E. coli M15 strain transformed with plasmid pZT32 (Japanese Patent Application No. 63-226288) was cultivated in an amount of 20 l using a 30 l Jarfermenter* (produced by Hitachi Seisakusho).

The following medium was used.

	Na ₂ HPO ₄ · 12H ₂ O	1.8%
10	KH ₂ PO ₄	0.2%
	(NH ₄) ₂ SO ₄	0.2%
	Yeast extract	0.5%
	Pepton*(Difco)	0.5%
	MgSO ₄ · 7H ₂ O	0.01%
15	Glucose	0.5%
	Ampicillin	150 µg/ml

500 ml of fungus liquid, which had been pre-cultivated on an LB-medium (T. Maniatis et al.; Molecular Cloning p48 (1982)) containing 150 µg/ml of ampicillin at 30°C overnight, was transferred on 500 ml of the above-mentioned medium, and then cultivated at 30°C. The cultivation was continued while ventilating air at 1 vvm and adjusting the pH of the medium to 7.0 with sodium hydroxide. When it was cultivated for 3 hours, OD₆₆₀ became 1, whereby IPTG was added in a concentration of 1 mM. The cultivation was continued for 6 more hours, whereby OD₆₆₀ reached 10, and the fungi were collected by centrifugal separation. After being washed with sterilized water, the fungus bodies were suspended in 10 mM Tris-HCl buffer (pH 8.0)/1 mM EDTA/0.1 mM DTT, and were homogenized by using a homogenizer 15HR (produced by Goring) at 10°C. The supernatant obtained by centrifugal separation was taken as a cell extract solution.

Using β-galactosidase as an index, purification of human calcitonin-fused polypeptide was carried out.

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First, low molecular proteins, etc., were removed by ultrafiltration (product name: Pelican* cassette) using a Millipore type PT filter (fractionation molecular weight = 300000), and then the extract was further purified by ion-exchange chromatography using a DEAE-TOYOPARL* 650C (produced by Tosoh). As the eluent buffer, 10 mM Tris-HCl buffer (pH 7.4)/0.1 mM EDTA/0.1 mM DTT was used. When non-adsorbed proteins were eluted (1000 ml), adsorbed proteins were eluted by a gradual concentration gradient of sodium chloride. The concentrations of sodium chloride at this time were 0.16 M, 0.32 M, and 0.8 M. The elution pattern is shown in Fig. 4. In the Figure, the concentration of sodium chloride is shown as _____. In Fig. 4, the β -galactosidase activity measured according to Miller's method (Miller. J., Experiments in molecular genetics 352-355 (1972)) is shown as $\circ\text{---}\circ$, and the amount of protein measured at an absorbency of 280 nm is shown as -----. The activity peaks were observed at a region of 1800-4500 ml of 0.32 M Sodium chloride eluted fractionation. Consequently, this eluted fractionation was defined as the purified protein fractionation. The amount of protein was measured according to Lowry's method (Lowry, O. H. et al., J. Biol. Chem., 193, 265 (1951)). The calibration curve for Lowry's method was prepared by using a bovine-serum albumin (produced by Sigma, Fraction V).

Here, 1 unit of β -galactosidase was defined as a titer in which it works on o-nitrophenol β -D-galactoside at pH 7.0 at 28°C to liberate 1 nmol of o-nitrophenyl for 1 minute.

Behaviors of specific activities by the above-mentioned treatment are shown in Table 1.

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Table 1

Step	Total Protein Amount (mg)	β -Galactosidase (U/mg)	Yield (%)
Cell extract	42200	63500	100
Ultrafiltration	22800	76900	65
DEAE Toyoparl 650C	9100	222000	39

10 The specific activity was increased about 3.5 times and was 222,000 U/mg protein.

Preparation of Crude Polypeptide by Specific decomposition of Fused Polypeptide

15 The above-mentioned human calcitonin-collagenase cleavage portion peptide- β -galactosidase fused polypeptide was specifically decomposed by using collagenase to obtain a C-terminal glycine adduct of human calcitonin. The collagenase used was available from Sigma*(Type VII). The composition of the reaction solution is shown as follows:

20 5 mM Calcium chloride
 50 mM Tris-HCl buffer, pH 7.5
 250 μ M Zinc chloride
 10 mM Dithiothreitol
 10 mM 2-Mercaptoethanol
25 1 mg/ml Fused protein purified standard
 100 unit/ml Collagenase

30 An enzyme reaction was carried out at 37°C for 3 hours, and the reaction product was confirmed with HPLC. This reaction solution was designated as the "aqueous solution containing a crude polypeptide". The conditions of HPLC analysis were as follows:

35 By using CAPCELL PAK C₁₈ SG 300 (6 mm x 35 mm) (produced by Shiseido) as a column, using an aqueous 0.1% trifluoroacetic acid solution/0.085% trifluoroacetic acid acetonitrile solution as an eluent, and linearly increasing the concentration of the 0.085% trifluoroacetic acid acetonitrile solution

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to 60% over a period of 20 minutes at a flow rate of 1.5 ml/min., a calcitonin precursor was eluted at an acetonitrile concentration of about 40%. The detection wavelength at this time was 214 nm.

5 Example 1: Purification of Crude Polypeptide

To the above-mentioned reaction solution containing the crude polypeptide, formic acid was added to a 2% concentration, and the solution was stirred, after which it was left to stand for 30
10 minutes at 4°C. After confirming that impurities had been sufficiently removed, the solution was centrifuged at 12000 rpm for 10 minutes to obtain a supernatant. The HPLC elution pattern of the supernatant at this time is shown in Fig. 1 (a). A
15 filter-paper (produced by Toyo Roshi, No.2) was placed on a magnet Buchner funnel, and 10 g of CAPCELL PAK C₈SG 300 powder (produced by Shiseido) was placed thereon, and the funnel was placed on a suction bottle. The supernatant was gently poured into the
20 above-mentioned Buchner funnel under suction. The HPLC elution pattern of the non-adsorbed fraction at this time is shown in Fig. 1 (b). After the suction was finished, the residue was washed with 50 ml of aqueous 0.1% trifluoroacetic acid solution (produced
25 by Wako Junyaku, for amino acid analysis) in two portions (HPLC elution pattern of the eluate; Fig. 1 (c)). It was then washed with 50 ml of aqueous 0.1% trifluoroacetic acid/20% acetonitrile solution in two
30 portions (HPLC elution pattern of the eluate; Fig. 1 (d)). Thereafter, the objective polypeptide was eluted with 5 ml of aqueous 0.1% trifluoroacetic acid/60% acetonitrile solution in ten portions (HPLC elution pattern of the eluate; Fig. 1 (e)); and finally the adsorbed substance was completely eluted
35 with methanol (for HPLC analysis: produced by Nakaraitesk). The results of the purification are shown in Table 2. The purity is shown as a percentage

by weight of human calcitonin precursor in the total protein. The purity after the treatment with formic acid was calculated from the sum of peaks I and II in Fig. 2. The purity was improved 56-fold by the formic acid treatment after the collagenase reaction, and 100% of human calcitonin precursor could be recovered. Also, the purity was further improved by more than 70% with the next treatment of CAPCELL PAK C₈ SG 300, attaining a 97% recovery.

Table 2

Step	Human Calcitonin precursor (mg)	Purity (%)	Yield (%)
Collagenase cleavage	120	0.9	100
Treatment with formic acid	120	51	100
Treatment with CAPCELL PAK C ₈ SG 300	115	>70	97

Fig. 2 is a drawing which shows an elution pattern when the eluate of Fig. 1 (e), after being freeze-dried, is analyzed with HPLC. As is clear from this figure, there are four strong peaks for the desired polypeptide in the elution pattern of HPLC analysis. They are due to the change of the N-terminal portion during the collagenase reaction. Each peak was analyzed by a peptide sequencer (produced by ABI, Model 471). According to the analysis, it was found that peaks I and II corresponded to human calcitonin precursors having 1 to 33 amino acids, peak III corresponded to that in which 1-7 positions in the N-terminal were deleted, and peak IV corresponded to that in which 1-8 positions in the N-terminal were deleted. In addition, peak II was found to correspond to that in which the S-S bonds in 1- and 7-positions had been reduced.

Analytical results, after which both the purification process of the present invention and an HPLC dispensing procedure were carried out, are shown in Fig. 3.

5 On the other hand, using the above-mentioned reaction solution containing the crude polypeptide, a purification of polypeptide was carried out by a conventional ion-exchanging column and HPLC as a comparative example. In this case, ten stages were
10 required for the purification. Each stage required 2 hours, and therefore, a total of 10 times the required period of Example 1 according to the present invention was required.

Examples 2 and 3

15 According to the expression of the gene coding for human calcitonin-collagenase cleavage portion peptide- β -galactosidase fused polypeptide by E. coli, and the purification procedure of Example 1,
20 angiotensin II-collagenase cleavage portion peptide- β -galactosidase fused polypeptide, and melanocyte-stimulating hormone-collagenase cleavage portion peptide- β -galactosidase fused polypeptide were produced and an aqueous solution containing a polypeptide was prepared in each case. These
25 solutions were treated in the same manner as that of Example 1. The HPLC elution patterns of eluates for angiotensin II and melanocyte-stimulating hormone are shown in Fig. 5 and Fig. 6, respectively.

[Industrial Applicability]

30 The process of the present invention can be advantageously carried out in any purification stage in the production of various polypeptides, especially physiologically active polypeptides.

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CLAIMS

1. A process for purifying a polypeptide, which comprises

(a) regulating the pH range of an aqueous solution containing a crude polypeptide to 1-4, to cause impurities to precipitate, followed by removing these impurities, and

(b) adsorbing the supernatant obtained in the above-mentioned stage (a) on a packing material for reversed phase high performance liquid chromatography, followed by eluting desired polypeptides, the adsorption step is carried out either by (1) mixing the supernatant and the packing material and putting the mixture into a Buchner funnel or by (2) putting the packing material into a Buchner funnel and then pouring the supernatant in to the Buchner funnel.

2. A process according to claim 1, wherein said crude polypeptide is a fused polypeptide which is cleaved into objective physiologically active moieties and other protein portions fused thereto.

3. A process for purifying a polypeptide, which comprises the steps of:

(a) regulating the pH range of an aqueous solution containing a crude polypeptide to 1-4 using formic acid, to cause impurities to precipitate, followed by removing these impurities leaving a supernatant,

wherein said crude polypeptide is a reaction solution in which a fused polypeptide is cleaved into physiologically active moieties having a molecular weight of not more than 15, 000 and another protein moiety fused thereto, wherein said physiologically active moiety is selected from the group consisting of insulin, growth hormone release factor, epidermal growth factor, atrial natriuretic peptide, thymosin α_1 , thymosin β_4 , thymopoietin transforming growth factor, adrenocorticotrophic hormone, calcitonin gene-related peptide, and cartilage factor;

and said other protein moiety is selected from the group consisting of β -galactosidase and chloramphenicol acetyltransferase; which is directly followed by

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(b) adsorbing the supernatant on a packing material for reversed phase high performance liquid chromatography by pouring said supernatant into a Buchner funnel into which said packing material has been placed, followed by eluting the desired polypeptides.

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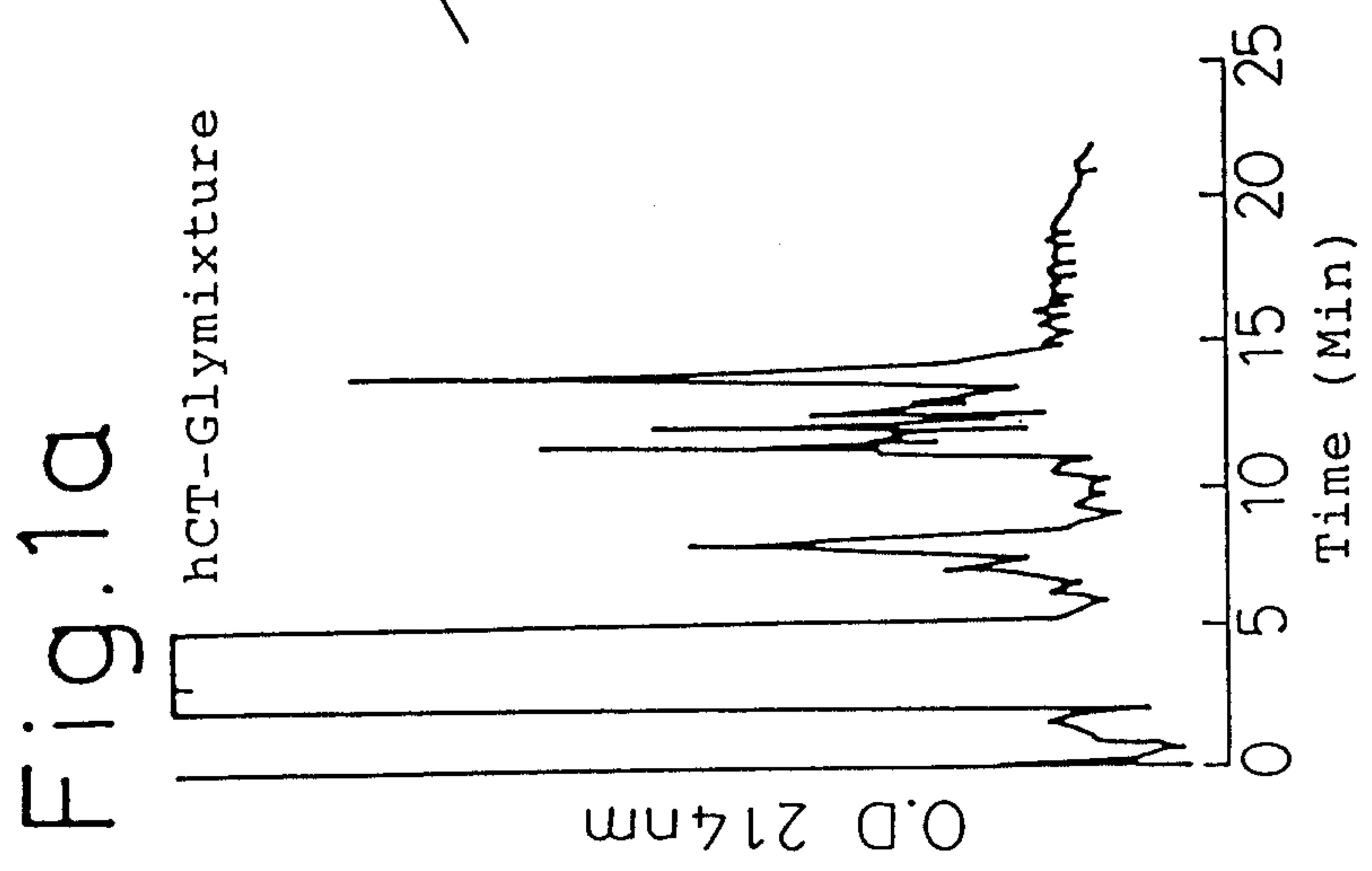
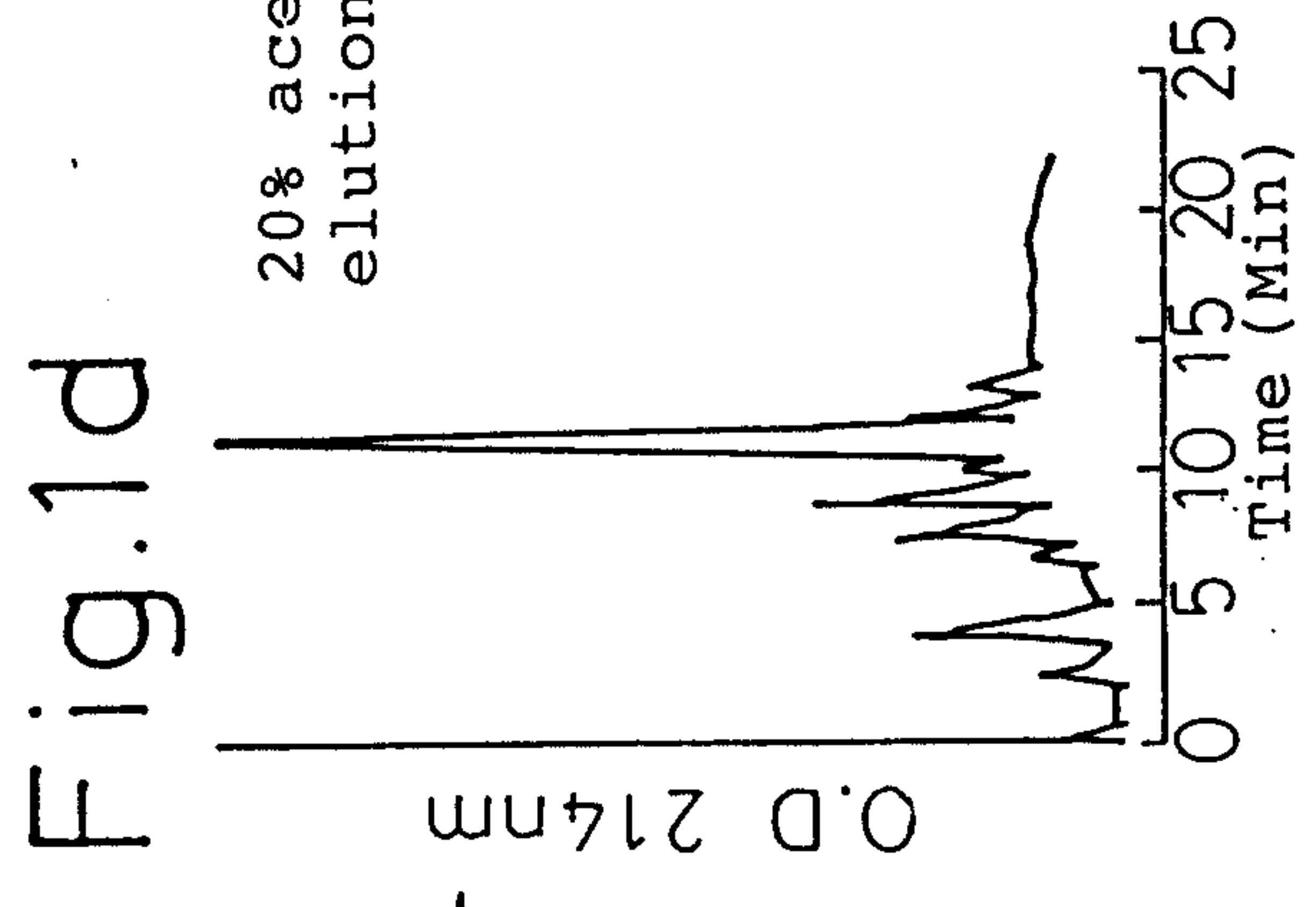
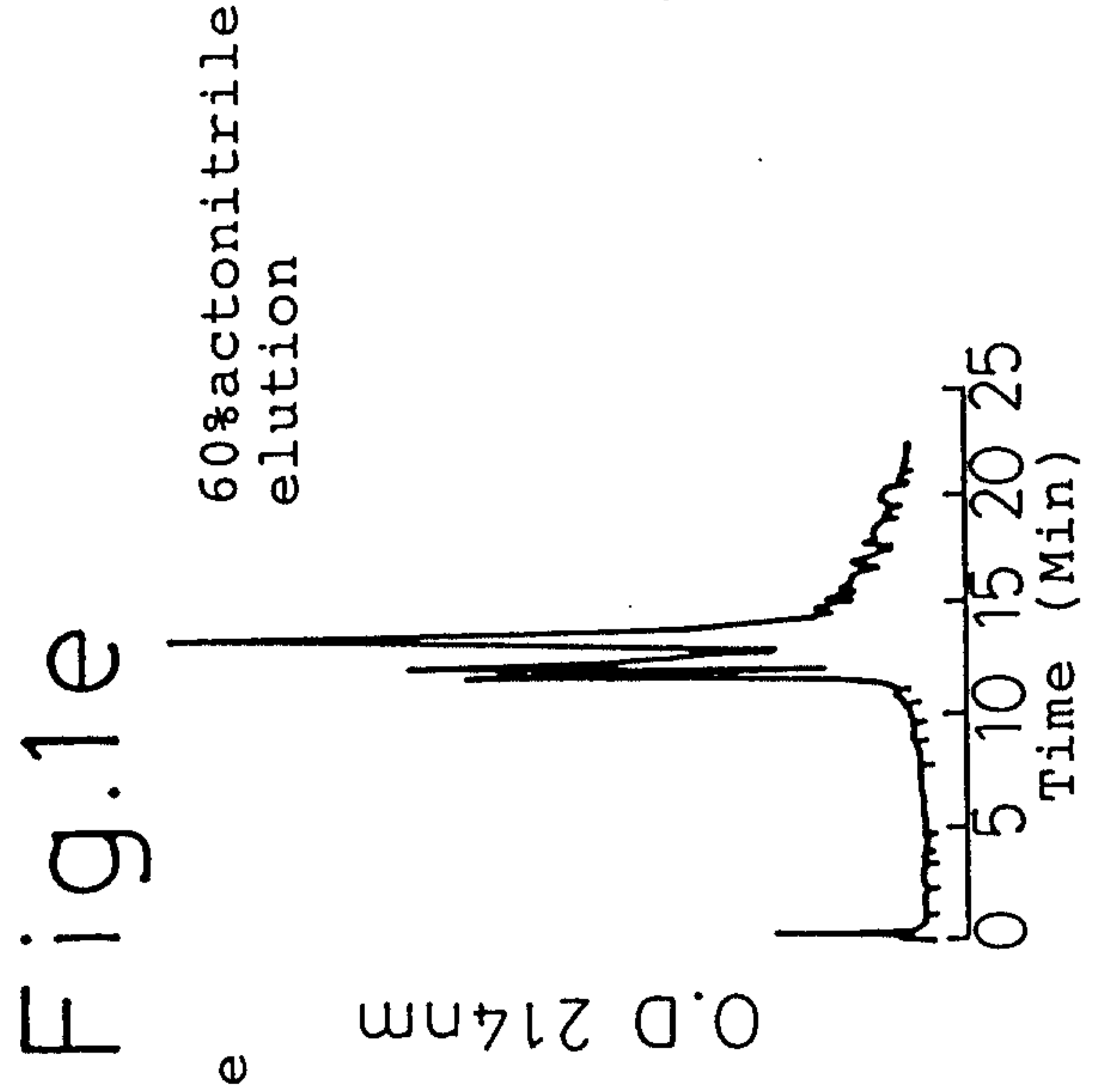
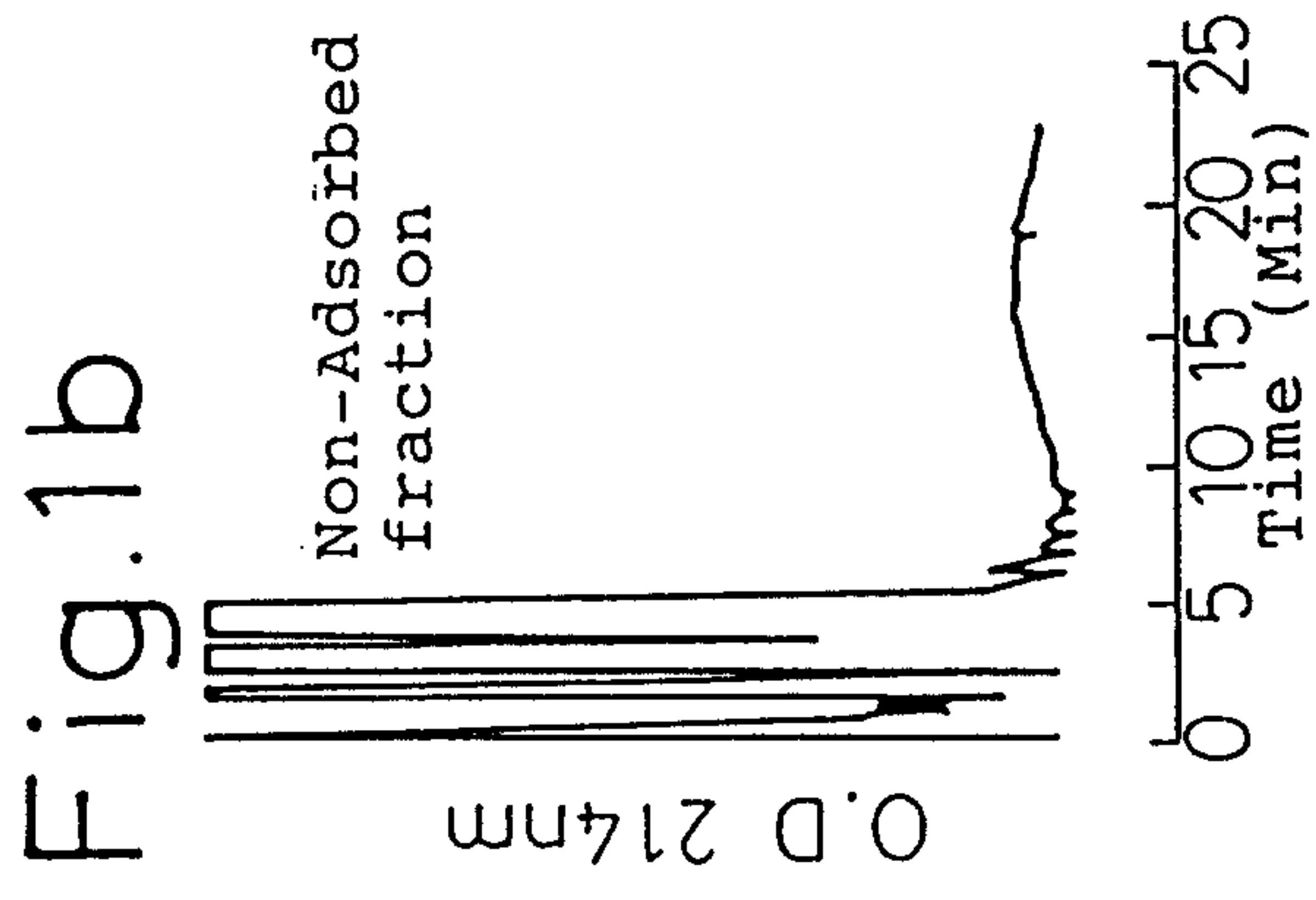
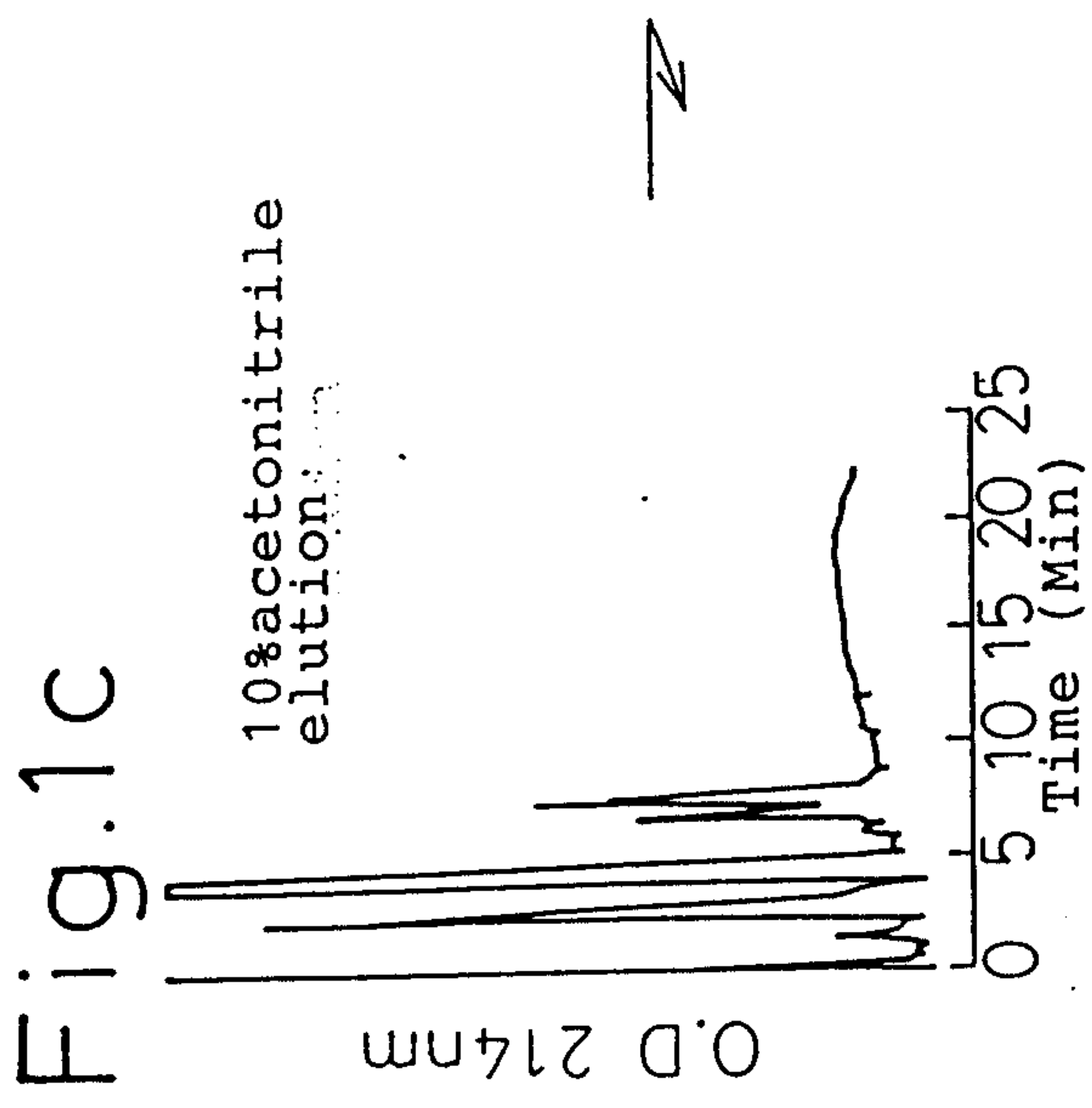
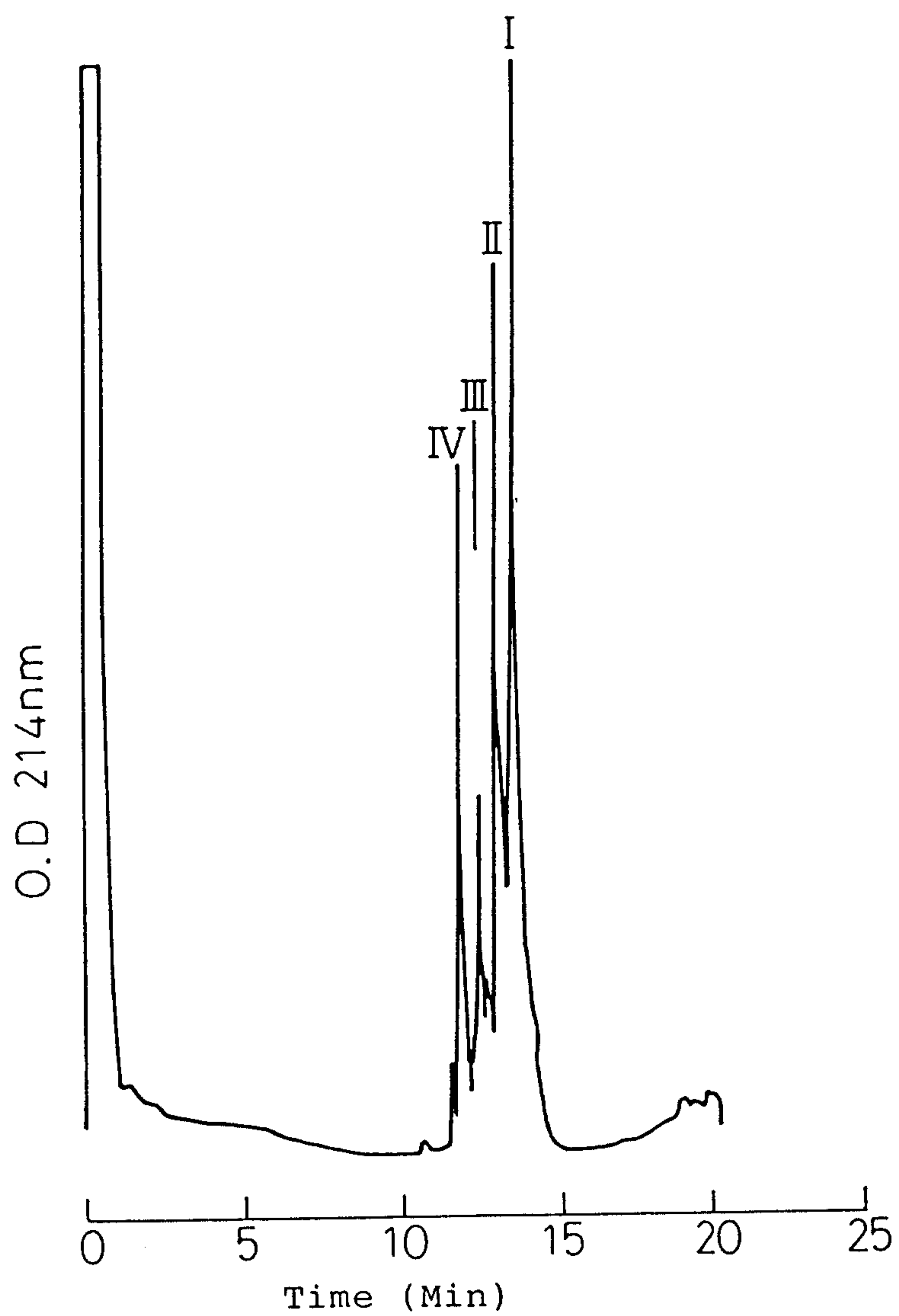


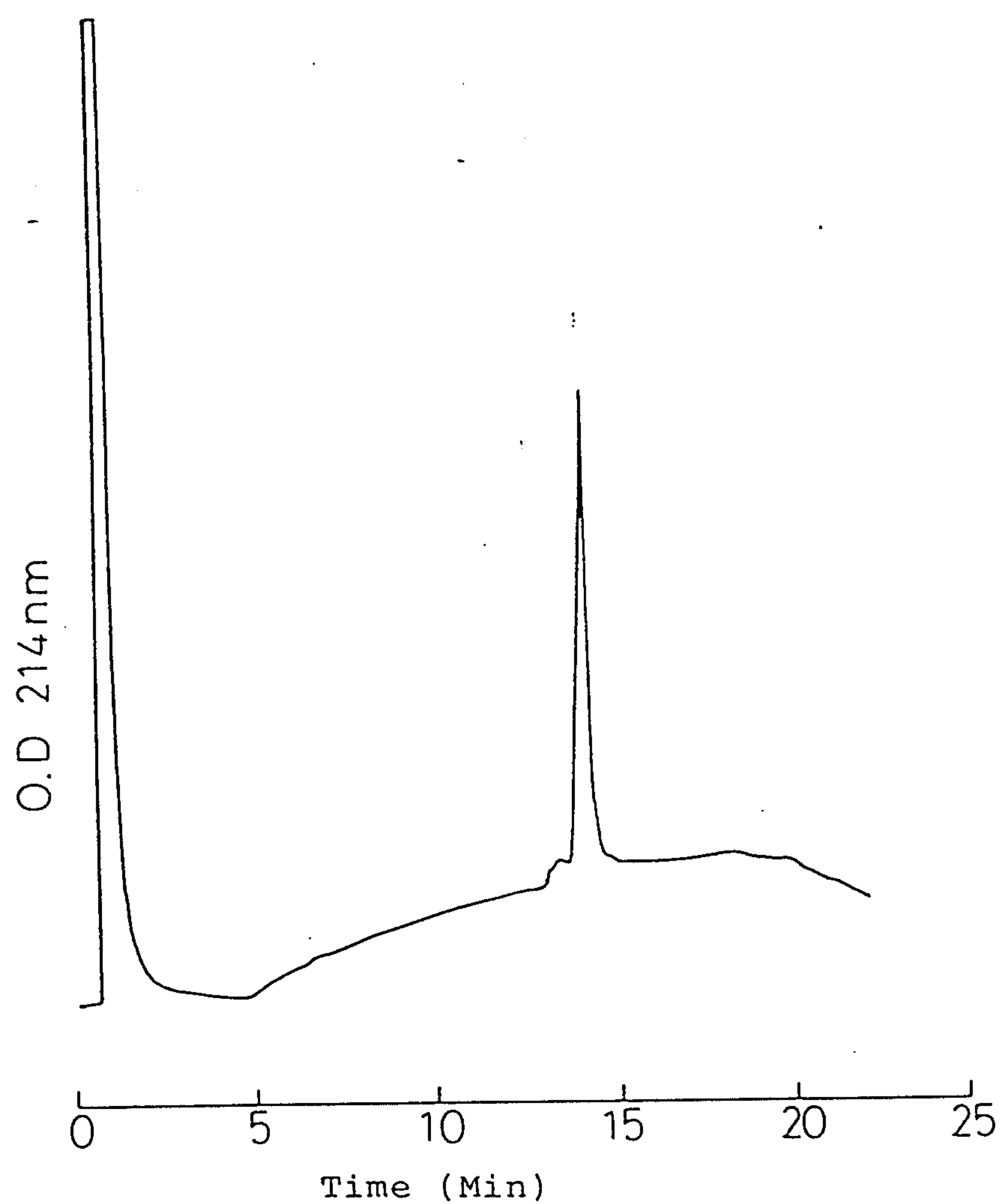
Fig.2



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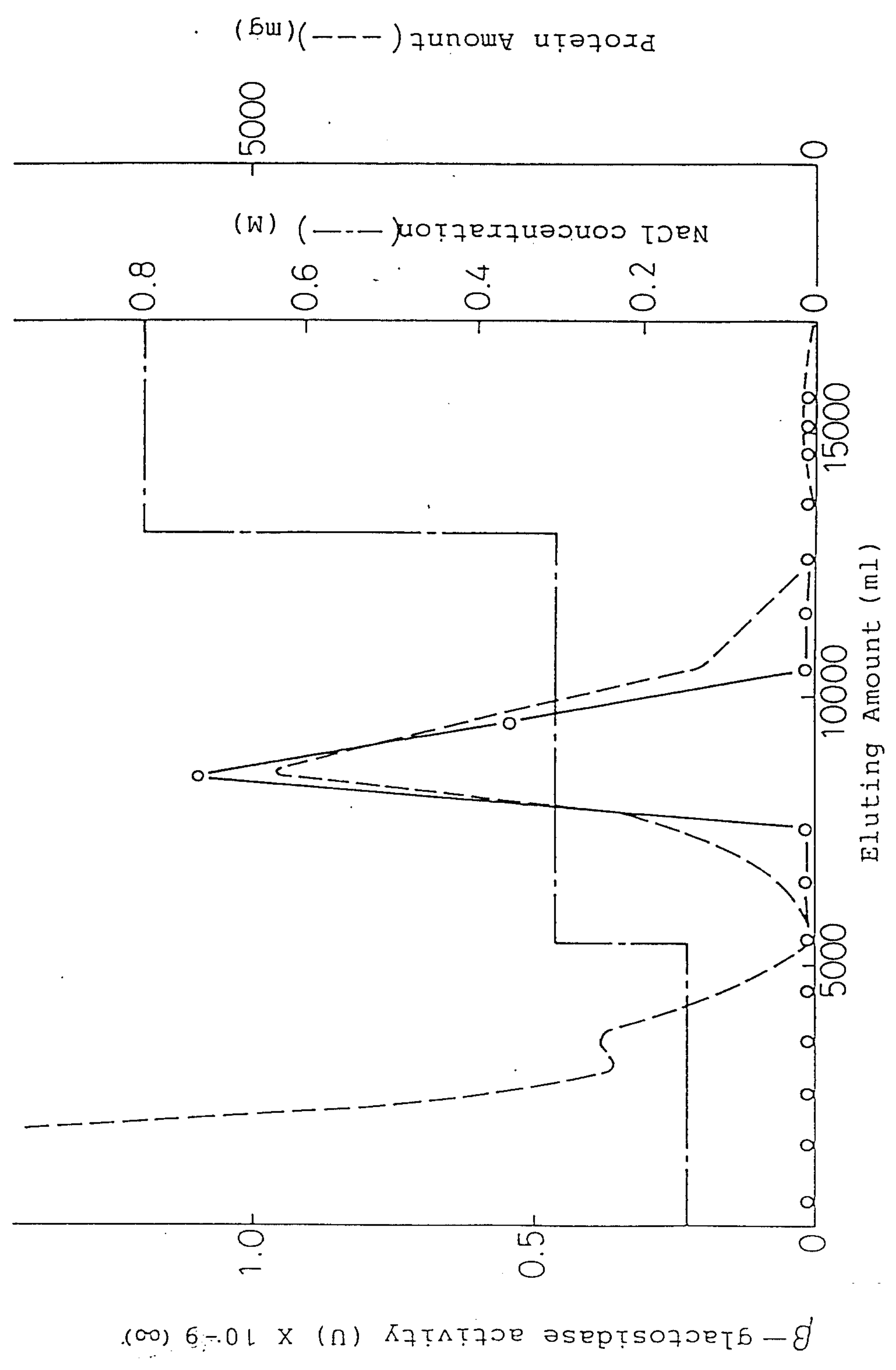
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Fig.3



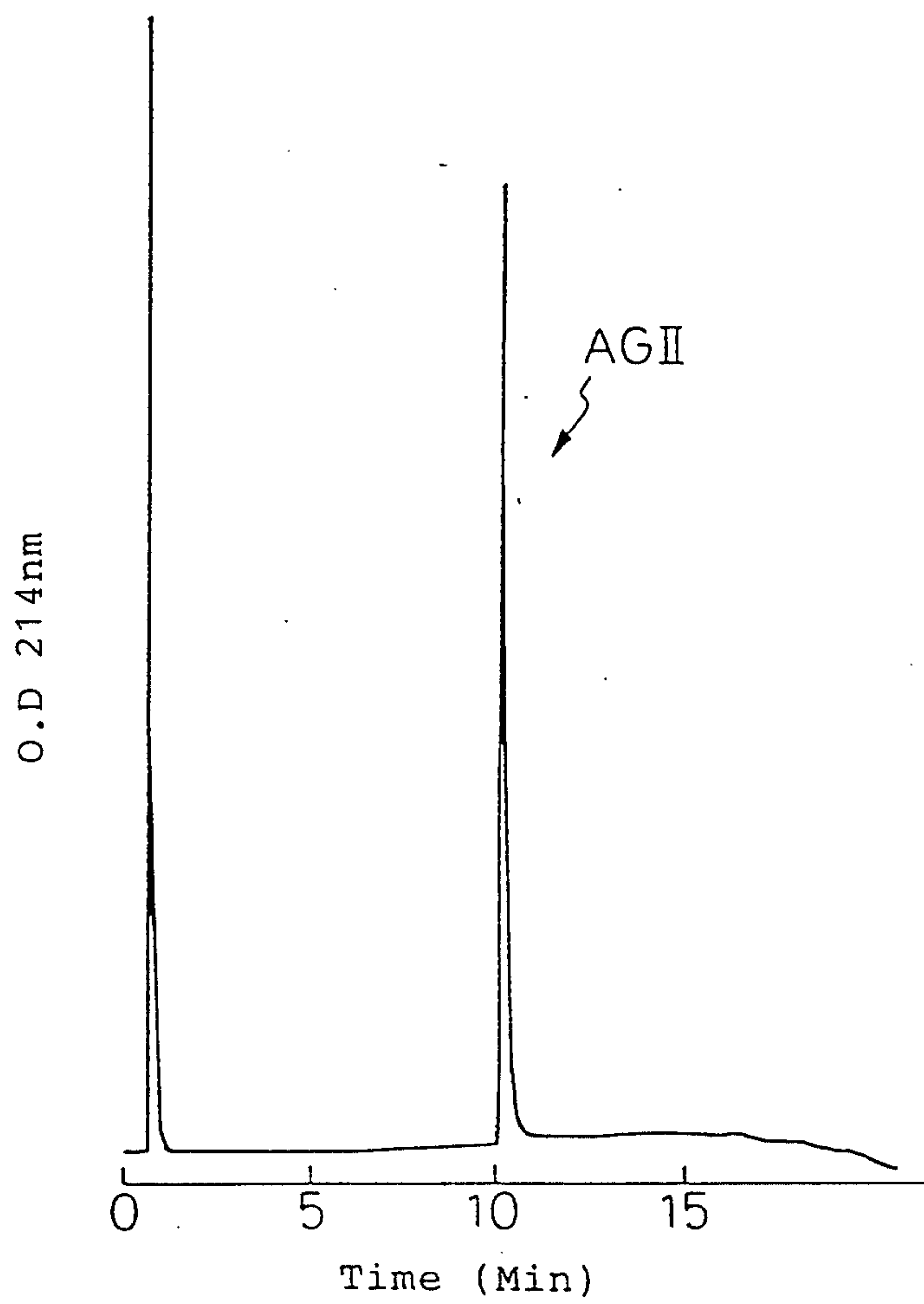
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Fig.4



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Fig.5



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Fig.6

