PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 1/113, 14/61 | A1

(11) International Publication Number: **WO 00/02900**

(43) International Publication Date: 20 January 2000 (20.01.00)

(21) International Application Number: PCT/SE99/01222

(22) International Filing Date: 5 July 1999 (05.07.99)

(30) Priority Data:
9802454-0 8 July 1998 (08.07.98) SE

(71) Applicant *(for all designated States except US)*: PHARMACIA & UPJOHN AB [SE/SE]; S-112 87 Stockholm (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HEMMENDORFF, Barbro [SE/SE]; Drejarvägen 8, S-141 73 Huddinge (SE). CASTAN, Andreas [SE/SE]; Vapengatan 24, S-126 52 Hägersten (SE). PERSSON, Anders [SE/SE]; Kålsängsgränd 6 D, S-753 19 Uppsala (SE).

(74) Agents: TANNERFELDT, Agneta et al.; Pharmacia & Upjohn AB, S-112 87 Stockholm (SE).

(81) Designated States: AU, CA, IL, JP, NZ, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD FOR THE PRODUCTION OF RECOMBINANT PEPTIDES WITH A LOW AMOUNT OF TRISULFIDES

(57) Abstract

The invention relates to a method for the production of recombinant peptides with a low amount of trisulfides which is characterized by the addition of a metal salt during or after the fermentation step and to a method for reduction of the amount of trisulfides in the production of recombinant peptides, characterized by the addition of a metal salt during or after fermentation. The peptide is preferably human growth hormone and the salt preferably a potassium or sodium phosphate.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Method for the production of recombinant peptides with a low amount of trisulfides.

The invention relates to a method for the production of recombinant peptides with a low amount of trisulfides which is characterized by the addition of a metal salt during or after the fermentation step and to a method for reduction of the amount of trisulfides in the production of recombinant peptides, characterized by the addition of a metal salt during or after fermentation. The peptide is preferably human growth hormone and the salt preferably a potassium or sodium phosphate.

Background

In the recombinant production of peptides, especially in the production of pharmaceuticals, the amount of contamination, such as variants of the wanted protein, should be reduced as much as possible both from economical and therapeutical aspects.

In the recombinant production of peptides, variants with an extra sulfur atom in a disulfide bridge sometimes are found, and the present invention relates to this problem.

Human Growth hormone, hGH, is a protein consisting of a single chain of 191 amino acids. The molecule is cross-linked by two disulfide bridges and the monomeric form has a molecular weight of 22 kDa.

hGH preparations have been prepared from human pituitaries, but nowadays the products on the market are produced by recombinant methods, rhGH.

Two types of therapeutically useful recombinant hGH preparations are present on the market: the authentic one, e.g. Genotropin®, Pharmacia & Upjohn AB, and an analogue with an additional methionine residue at the N-terminal end, e.g. Somatonorm®.

hGH is used to stimulate linear growth in patients with hypo pituitary dwarfism or Turner's syndrome but other indications have also been suggested.

A new variant of human growth hormone, hGH, has been found and reference is given to Pavlu et al, 1993, Bioseparation 3, 257-265. This variant has been identified and characterized, see Andersson et al, 1996, Int. J. Peptide, Protein,. Res. 47, 311-321. The

variant, which is formed during the expression of hGH in Escherichia coli, is found to be more hydrophobic than rhGH and has been structurally defined as a trisulfide variant of rhGH. The variant is only formed during synthesis in E Coli and has not been found in hGH preparations from human pituitaries.

This phenomenon of the trisulfides in peptides, produced by recombinant methods, has also been described for recombinant superoxide dismutase (Briggs et al, 1987, Biochem., Biophys. Acta, 537, 100-109) and for a mutein of interleukin, (Breton J et al. J. Chromatogr. A., 1995, 709(1), 135-46).

In WO 94/24127 a method for converting a hydrophobic derivative of a growth hormone into the native form of growth hormone is disclosed. The hydrophobic derivative of the growth hormone comprises an extra sulfur atom. The method is a chemical treatment of the derivative of growth hormone with a mercapto compound. As examples are cystein, gluthatione, 2-mercapto ethanol and dithiothreitol given.

In WO 96/02570 a method is disclosed comprising the chemical treatment with a sulfite compound for the conversion of the derivative of growth hormone into the native form. Mercapto compounds and sulfite compounds are used in the redox-reaction for the conversion of the already formed growth hormone comprising an extra sulfur atom.

The invention

We have now found a new method for the reduction of the amount of trisulfides in the production of recombinant peptides, e.g. both proteins and smaller peptides.

The invention is based on the novel and unexpected finding that the amount of trisulfides in the production of recombinant peptides can be reduced by the addition of a metal salt, preferably in excess, already during or after fermentation and not, as earlier suggested, by conversion of the formed trisulfide of growth hormone into the native form.

This reduced amount of the derivative is due to inhibition of the activity of H_2S in the medium and the prevention of the formation of the modified growth hormone comprising an extra sulfur atom

The addition can be done directly after fermentation, e.g. after the fermentation has been terminated and the cells are harvested and before further process steps.

The addition can e.g. be done with a buffer including the salt.

The protein can be any recombinant protein but is preferably recombinant growth hormone which can be both human and animal such as human growth hormone (hGH), bovine growth hormone (bGH) and porcine growth hormone (pGH).

The metal salt can be any metal chosen among alkalimetal and earth metal.

pH is preferably equal to or lower than pH 7. More preferable pH is equal to or lower than 6.8 and most preferable pH is equal to or lower than 6.0.

The pH regulation can be achieved with a selected buffer including the metal salt.

The metal is preferably alkali, such as sodium or potassium and the salt is preferably sodium or potassium phosphate or acetate.

The concentration of free sulfide ions is minimized by addition of the metal salt in molar excess.

The used metal salt is preferably not a sulfite or a mercapto compound.

The attached claims define the invention.

Figure 1 shows the amount of trisulfide-GH in the extracts.

Figure 2 shows the induction and inhibition of trisulfide formation in GH

In the examples below a recombinant produced hGH has been produced or used, but the invention as claimed is not limited to this peptide. The trisulfide variant is named trisulfide-GH.

EXAMPLES

hGH was produced in *E. Coli* according to known methods. Reference can be given to EP 177343, example 8.

The transformant of *E. Coli* was fermented in the medium, the culture was agitated under aeration and glucose was added. The fermentation was terminated by turning off the glucose and aeration. At this point a reference sample was taken. Thereafter the cells were harvested.

For the production of pure hGH, the harvested cells were concentrated, washed, solubilized by freezing, thawed and purified according to known methods.

Example 1. pH variation, lab scale.

The culture was harvested and the cells were concentrated by microfiltration. The pH was 7.3 in the cell concentrate. Four batches of the cell concentrate were taken. In three batches (500 ml) the pH was adjusted to 6.5, 7.0 and 7.8, with HCl or NaOH, respectively. The fourth batch is the non-treated comparison sample. Thereafter the cell concentrates were frozen.

The four batches were thawed and the cell concentrates were diluted twice with a buffer containing 10 mM Tris-HCl and 1 mM Na_2-EDTA pH 8.2. Cell free extracts were obtained by centrifugation.

The amount of trisulfide-GH in the extracts was determined.

The result is shown in Figure 1.

It was found that the amount of trisulfide-GH was highest at pH 7.8 (12%). This could be compared to the fourth batch which was not pH-changed.

A pH above 7.0 gave too high amount of trisulfide-GH in this experiment, thus pH should be lower.

Example 2. Pilot scale

The culture was harvested and the cells were concentrated by microfiltration. The pH in the cell concentrate was 7.2. The cell concentrate was divided in two portions (about 30 L).

Cell concentrate A was washed with about one volume of water and was thereafter frozen at - 30°C.

Cell concentrate B was washed with about one volume of 0.05 M potassium phosphate buffer, pH 6.6. The pH in cell concentrate B was 6.8. The cell concentrate was thereafter frozen at-30°C.

After thawing, the concentrated cells were extracted by diafiltration with Tris-HCl /EDTA buffer and the amount of trisulfides was determined. The amount of trisulfide-GH was 6% in extract A and about 3 % in extract B, thus the double in A compared to B. This showed that low pH and the metal salt buffer reduces the amount of the trisulfide variant of growth hormone.

Example 3. Pilot scale

The amount of trisulfides in the reference sample, taken before harvest, was determined. The culture was harvested and the cells were concentrated by microfiltration. The pH in the cell concentrate was 7.2. The cell concentrate was divided in two portions (about 30 L). Cell concentrate C was washed with about one volume of water and was thereafter frozen at - 30°C.

Cell concentrate D was washed with about one volume of 0.9 % NaCl in water. The pH in that cell concentrate was 7.2. The cell concentrate was thereafter frozen at-30°C.

After thawing, the concentrated cells were extracted by diafiltration with Tris-HCl /EDTA buffer and the amount of trisulfides was determined. The amount of trisulfide-GH was about 5 % in extract C and about 4.8 % in D, thus the same in C and D. The ratio of trisulfide-GH in extract C : reference sample was 5.0 % : 2.0 % = 2.5 and the ratio of trisulfide-GH in extract D : reference sample was 4.7 % : 2.0 % = 2.4

This showed that for a periplasmatic extract not only the addition of a metal salt but also the low pH is of importance.

Example 4. Pilot scale

The amount of trisulfides in the reference sample, taken before harvest, was determined. The culture was harvested and the cells were concentrated by microfiltration. The pH in the cell concentrate was 7.2. The cell concentrate (E) was washed with about one volume of 0.025 M sodium phosphate buffer pH 6.0, to which 1 ml/L HCl 37 % was added. The pH in cell concentrate E was 5.9. The cell concentrate was thereafter frozen at-30°C.

After thawing the concentrated cells were extracted by diafiltration with Tris-HCl /EDTA buffer and the amount of trisulfides was determined.

The ratio of trisulfide-GH in extract E : reference sample was 1.6 % : 1.4 % = 1.1.

This showed that the amount of trisulfide-GH can be reduced by the addition of a metal salt and a low pH.

Example 5. Pilot scale

The amount of trisulfides in the reference sample, taken before harvest, was determined. The culture was harvested and the cells were concentrated by microfiltration. The pH in the cell concentrate was 7.2. The cell concentrate was divided in two portions (about 30 L).

Cell concentrate F was washed with about one volume of acetate buffer, containing sodium acetate x $3H_2O$, 8.03 g/L and acetic acid (100 %) 2.35 ml/L. The pH in cell concentrate F was 5.9.The cell concentrate was thereafter frozen at - 30°C.

Cell concentrate G was washed with about one volume of 0.025 M sodium phosphate buffer pH 6.0, to which 0.5 ml/L concentrated H_2SO_4 was added. The pH in cell concentrate G was 5.9. The cell concentrate was thereafter frozen at-30°C.

After thawing the concentrated cells were extracted by diafiltration with Tris-HCl /EDTA buffer and the amount of trisulfides was determined.

The ratio of trisulfide-GH in extract F : reference sample was 3.4 % : 3.1 % = 1.1 and the ratio of trisulfide-GH in extract G : reference sample was 2.6 % : 3.1 % = 0.8.

This showed that the amount of trisulfide-GH can be reduced by the addition of a metal salt and a low pH.

Example 6. Comparison of buffers and pH.

250µl of pure hGH (from the production of Genotropin®) in water (2.436 mg/ml) + 250µl of different 100 mM buffers , see Table 1, were mixed. Saturated H_2S (0.11 M) in distilled water was used immediately after preparation. 50µl of distilled water (control) or H_2S in three different dilutions was added to each sample. (0.5, 0.1 and 0.02 mM H_2S, respectively)

The concentration was thereafter 1.11 mg hGH/ml.

These solutions were incubated with the different concentrations of H_2S during 3 hours at room temperature for the preparation of the trisulfide variant of hGH.

After incubation, freezing, thawing and desalting of all samples in 25 mM Tris-HCl at pH 7.6, the amount of trisulfide was analyzed.

The buffers were prepared according to standard tables.

Table 1

Na-phosphate, pH 7.8

Na-phosphate, pH 7.0

Na-phosphate, pH 6.5

Na-phosphate, pH 6.0

Na-citrate, pH 6.2

Tris-HCl, pH 7.6

Ammonium citrate, pH 6.2

The result is shown in Figure 2.

Ammonium citrate gave no reduction of trisulfides despite the low pH.

Na-phosphate at pH 6.0 gave the best result but also Na-phosphate at higher pH can be used.

This showed that for pure hGH the addition of a metal salt is of importance for the amount of trisulfides.

CLAIMS

1. Method for the production of recombinant peptides with a low amount of trisulfides, characterized by the addition of a metal salt during or after the fermentation step.

2. Method for the reduction of the amount of trisulfides in the production of recombinant peptides, characterized by the addition of a metal salt during or after fermentation.

3. Method according to any of claims 1 to 2 in which the addition is performed directly after fermentation.

4. Method according to any of claims 1 to 3 in which the metal salt is chosen among alkali metals and earth metals.

5. Method according to any of claims 1 to 4 in which pH is equal to or lower than pH 7.

6. Method according to any of claims 1 to 5 in which the metal preferably is potassium or sodium.

7. Method according to claim 6 in which the salt preferably is potassium- or sodium phosphate or acetate.

8. Method according to any of claims 1 to 7 in which the peptide preferably is growth hormone and more preferably human growth hormone.

9. Use of a metal salt in the production of recombinant peptides during or after the fermentation step for the reduction of the amount of trisulfides in the recombinant product.

10. Use of metal salt for the reduction of the amount of trisulfides in the production of recombinant peptides by the addition of a metal salt during or after fermentation

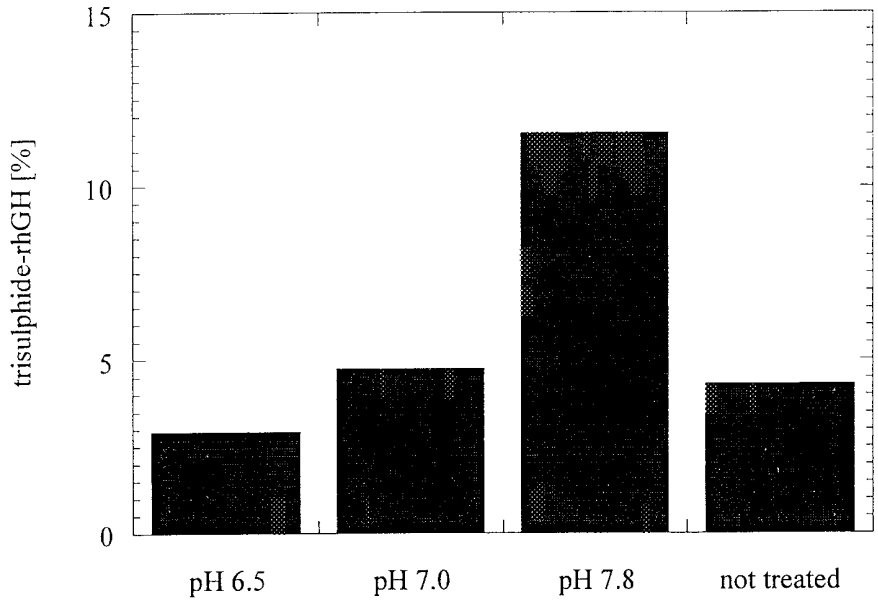


Figure 1

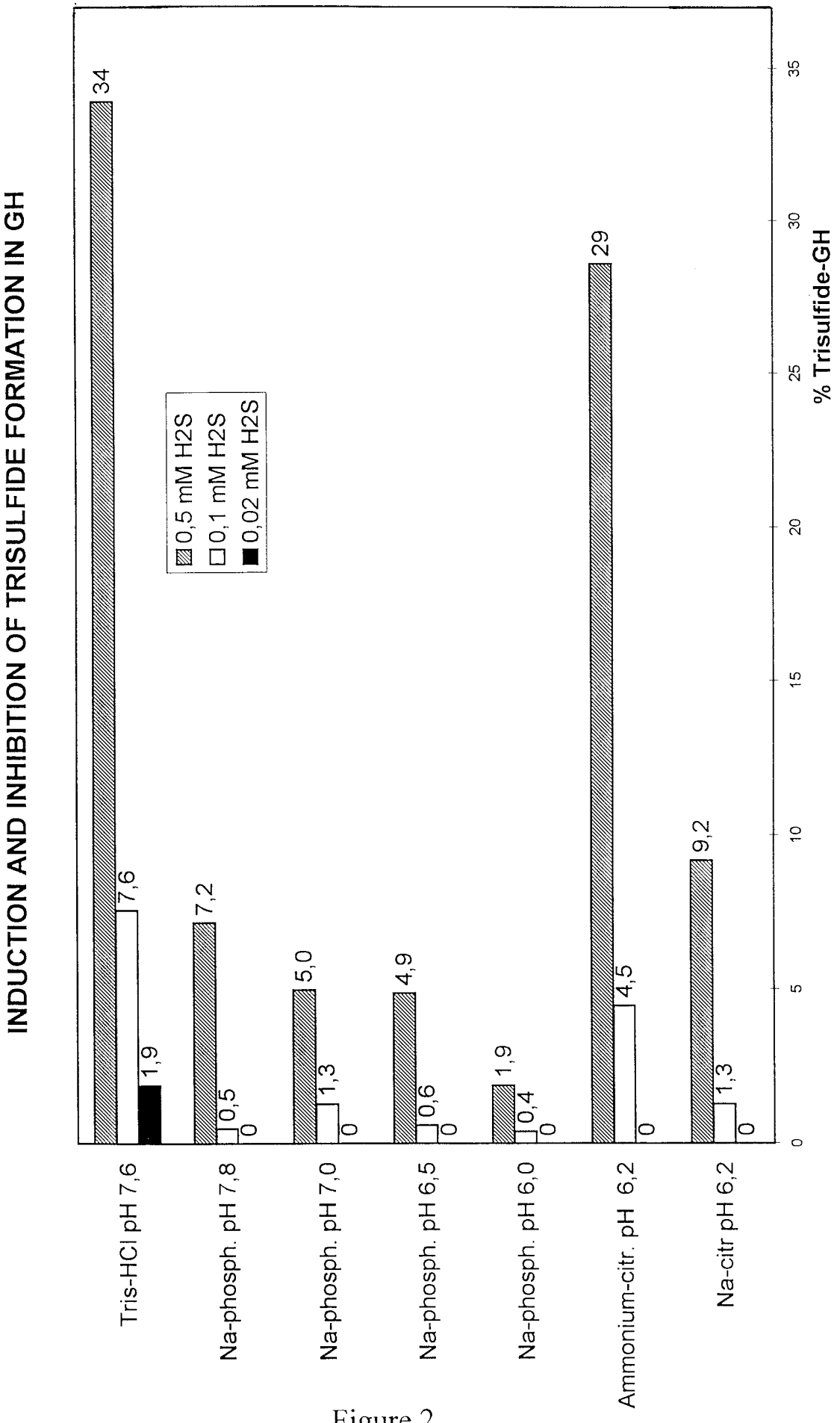

INDUCTION AND INHIBITION OF TRISULFIDE FORMATION IN GH
0,5 mM H2S
0,1 mM H2S
0,02 mM H2S
% Trisulfide-GH
0
5
10
15
20
25
30
35
34
7,6
1,9
7,2
0,5
0
5,0
1,3
0
4,9
0,6
0
1,9
0,4
0
29
4,5
0
9,2
1,3
0
Tris-HCl pH 7,6
Na-phosph. pH 7,8
Na-phosph. pH 7,0
Na-phosph. pH 6,5
Na-phosph. pH 6,0
Ammonium-citr. pH 6,2
Na-citr pH 6,2

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/01222

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 1/113, C07K 14/61
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9602570 A1 (NOVO NORDISK A/S), 1 February 1996 (01.02.96), see page 3, line 28 - page 5, line 17; claims ---	1-10
A	WO 9506064 A1 (GENENTECH, INC.), 2 March 1995 (02.03.95), see page 8, line 15 - page 9, line 16; claim 22 ---	1-10
A	WO 9424157 A1 (NOVO NORDISK A/S), 27 October 1994 (27.10.94), claims --- --------	1-10

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 Sept 1999	30 -10- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Hampus Rystedt/EÖ Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

30/08/99

International application No.
PCT/SE 99/01222

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9602570 A1	01/02/96	AU 2922095 A	16/02/96
		IL 114597 D	00/00/00
		ZA 9505789 A	11/03/96
WO 9506064 A1	02/03/95	CA 2168552 A	02/03/95
		EP 0714406 A	05/06/96
		JP 9501693 T	18/02/97
		US 5663304 A	02/09/97
		US 5756672 A	26/05/98
		US 5808006 A	15/09/98
WO 9424157 A1	27/10/94	AU 6535594 A	08/11/94
		BG 100068 A	31/12/96
		CA 2160663 A	27/10/94
		CZ 9502728 A	13/03/96
		EP 0695310 A	07/02/96
		FI 955000 A	19/10/95
		HU 73320 A	29/07/96
		HU 9503020 D	00/00/00
		IL 109347 D	00/00/00
		JP 8508735 T	17/09/96
		NO 954181 A	19/10/95
		PL 311198 A	05/02/96