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CA 2757637 C 2017/10/24

(11)(21) **2 757 637**

(12) **BREVET CANADIEN**
CANADIAN PATENT

(13) **C**

(86) **Date de dépôt PCT/PCT Filing Date:** 2010/04/23
(87) **Date publication PCT/PCT Publication Date:** 2010/11/04
(45) **Date de délivrance/Issue Date:** 2017/10/24
(85) **Entrée phase nationale/National Entry:** 2011/10/04
(86) **N° demande PCT/PCT Application No.:** EP 2010/055432
(87) **N° publication PCT/PCT Publication No.:** 2010/124998
(30) **Priorités/Priorities:** 2009/04/27 (US61/214,650); 2009/04/27 (EP09158788.1)

(51) **Cl.Int./Int.Cl.** ***G01N 33/569*** (2006.01)

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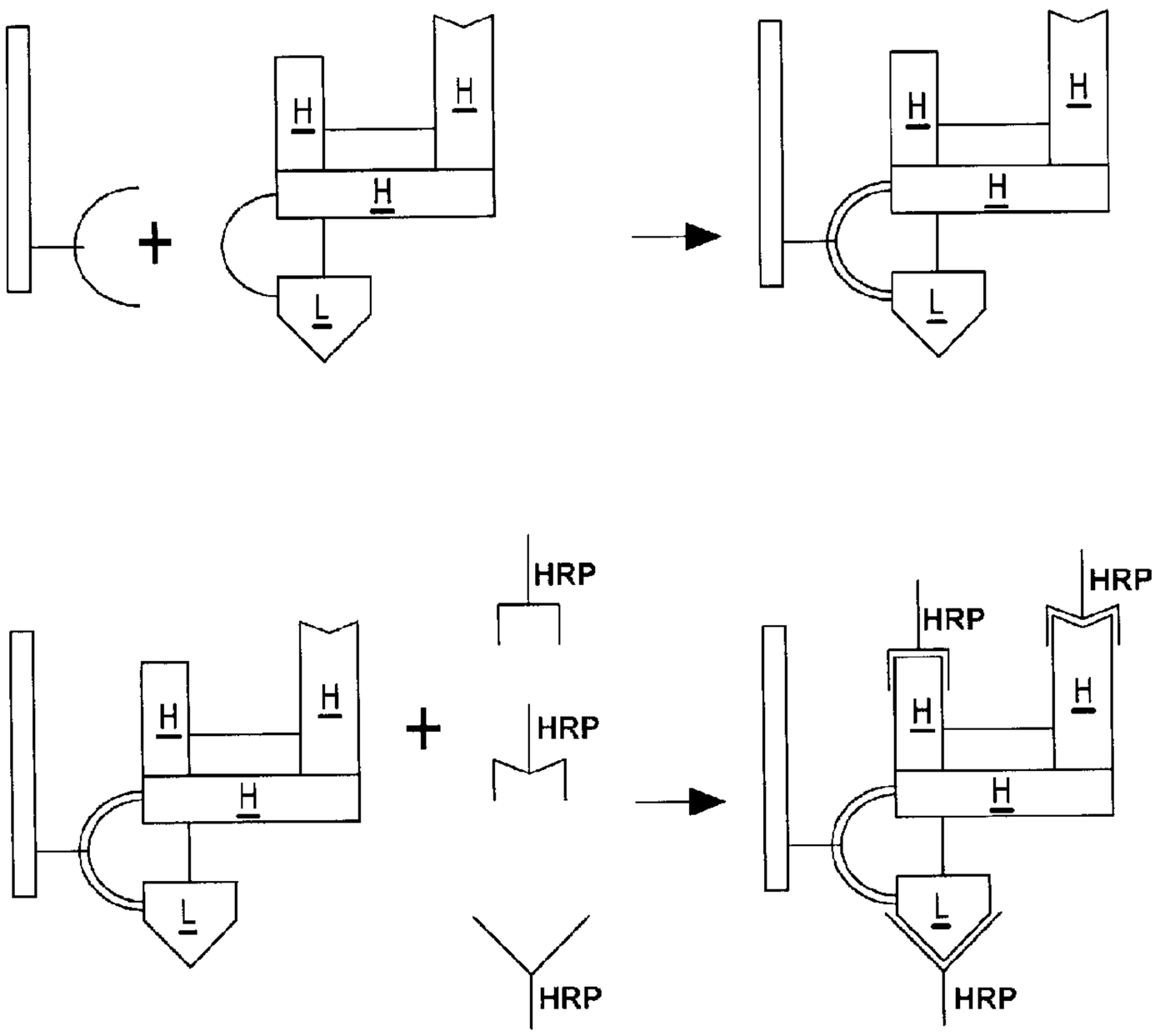
(54) **Titre : MOYENS ET PROCEDES POUR DETERMINER LA QUANTITE DE POLYPEPTIDE NEUROTOXINE ET DE SES ACTIVITES CATALYTIQUES ET PROTEOLYTIQUES**

(54) **Title: MEANS AND METHODS FOR THE DETERMINATION OF THE AMOUNT OF NEUROTOXIN POLYPEPTIDE AND OF ITS CATALYTIC AND PROTEOLYTIC ACTIVITIES**



(57) **Abrégé/Abstract:**
The present invention pertains to the field of tools for ensuring manufacture of polypeptides and quality control. Specifically, it relates to a method for determining of the amount of processed (active) Neurotoxin polypeptide in a solution comprising pro-cessed Neurotoxin polypeptide and partially processed or unprocessed Neurotoxin polypeptide. The present in-vention relates further to a device for determining said amount and a kit adapted to carry out the method of the present invention.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 November 2010 (04.11.2010)

PCT

(10) International Publication Number
WO 2010/124998 A1

(51) International Patent Classification:
G01N 33/569 (2006.01)

(21) International Application Number:
PCT/EP2010/055432

(22) International Filing Date:
23 April 2010 (23.04.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

09158788.1	27 April 2009 (27.04.2009)	EP
61/214,650	27 April 2009 (27.04.2009)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: MEANS AND METHODS FOR THE DETERMINATION OF THE AMOUNT OF NEUROTOXIN POLYPEPTIDE AND OF ITS CATALYTIC AND PROTEOLYTIC ACTIVITIES

(57) **Abstract**: The present invention pertains to the field of tools for ensuring manufacture of polypeptides and quality control. Specifically, it relates to a method for determining of the amount of processed (active) Neurotoxin polypeptide in a solution comprising processed Neurotoxin polypeptide and partially processed or unprocessed Neurotoxin polypeptide. The present invention relates further to a device for determining said amount and a kit adapted to carry out the method of the present invention.

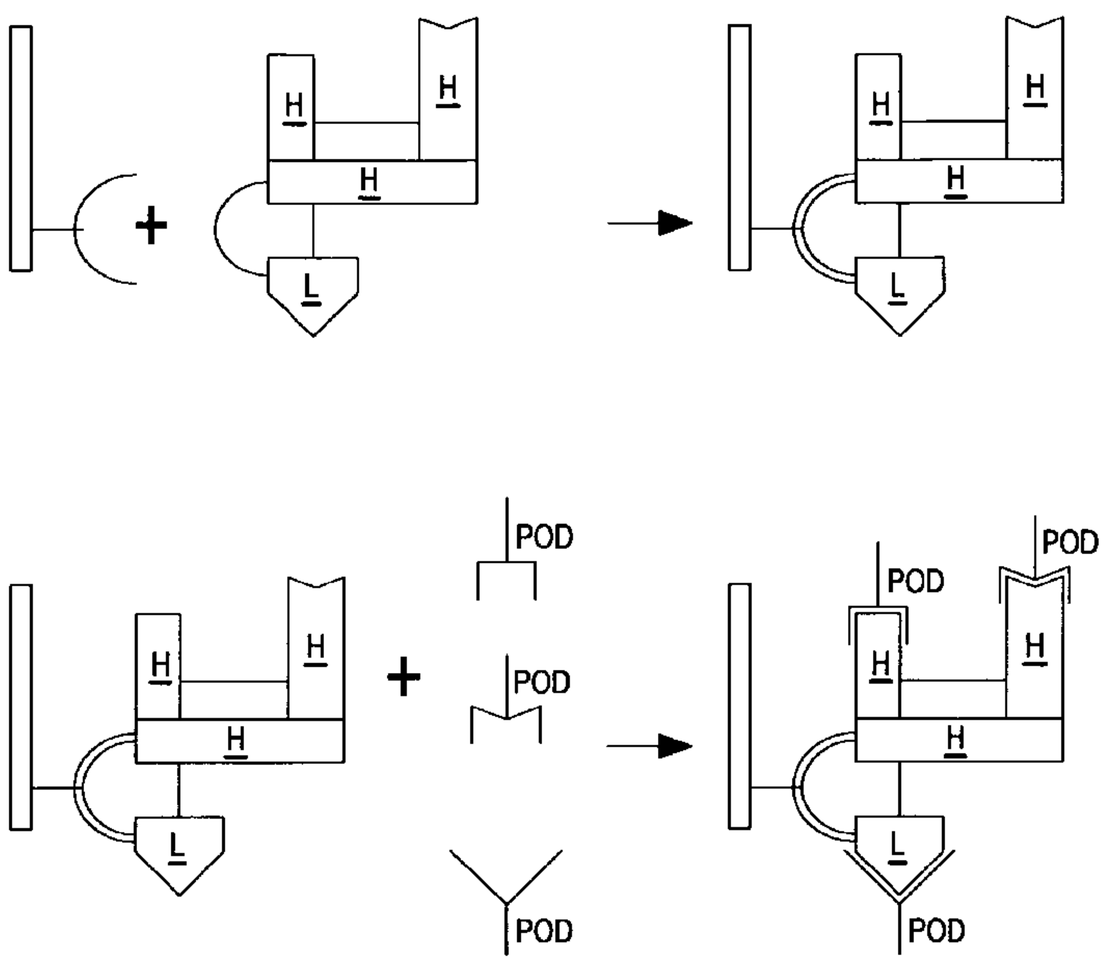


Fig. 2

WO 2010/124998 A1

Means and Methods for the determination of the amount of Neurotoxin polypeptide and of its catalytic and proteolytic activities

[0001] The present invention pertains to the field of tools for ensuring manufacture of polypeptides and quality control. Specifically, it relates to a method for determining of the amount of processed (active) Neurotoxin polypeptide in a solution comprising processed Neurotoxin polypeptide and partially processed or unprocessed Neurotoxin polypeptide. The present invention relates further to a device for determining said amount and a kit adapted to carry out the method of the present invention.

[0002] Clostridium botulinum and Clostridium tetani produce highly potent neurotoxins, i.e. botulinum toxins (BoNTs) and tetanus toxin (TeNT), respectively. These Clostridial Neurotoxins (CNTs) specifically bind to neuronal cells and disrupt neurotransmitter release. Each toxin is synthesized as an inactive unprocessed approximately 150 kDa single-chain protein. The posttranslational processing involves formation of disulfide bridges, and limited proteolysis (nicking) by the bacterial protease(s). Active Neurotoxin consists of two chains, an N-terminal light chain of approx. 50 kDa and a heavy chain of approx. 100 kDa linked by a disulfide bond. CNTs structurally and functionally consist of three domains, i.e. the catalytic light chain, the heavy chain encompassing the translocation domain (N-terminal half) and the receptor binding domain (C-terminal half), see Krieglstein 1990, Eur J Biochem 188, 39; Krieglstein 1991, Eur J Biochem 202, 41; Krieglstein 1994, J Protein Chem 13, 49. The Botulinum Neurotoxins are synthesized as molecular complexes comprising the 150 kDa Neurotoxin protein and associated non-toxic proteins. The complex sizes differ based on the Clostridial strain and the distinct Neurotoxin serotypes ranging from 300 kDa, over 500 kDa, and 900 kDa. The non-toxic proteins in these complexes stabilize the Neurotoxin and protect it against degradation, see Silberstein 2004, Pain Practice 4, S19 – S26.

[0003] Clostridium botulinum secretes seven antigenically distinct serotypes designated A to G of the botulinum neurotoxin (BoNT). All serotypes together with the related tetanus neurotoxin (TeNT) secreted by Clostridium tetani, are Zn2+-endoproteases that block synaptic exocytosis by cleaving SNARE proteins, see Couesnon, 2006, Microbiology, 152, 759. CNTs cause the flaccid muscular paralysis seen in botulism and tetanus, see Fischer 2007, PNAS 104, 10447.

[0004] Despite its toxic effects, botulinum toxin complex has been used as a therapeutic agent in a large number of diseases. Botulinum toxin serotype A was approved for human use in the United States in 1989 for the treatment of strabism, blepharospasm, and other disorders. It is commercially available as Botulinum toxin A protein preparation, for example, under the tradename BOTOX (Allergan Inc) or under the tradename DYSPORT (Ipsen Ltd). An improved, complex-free Botulinum toxin A preparation is commercially available under the tradename XEOMIN (Merz Pharmaceuticals GmbH). For therapeutic applications, the preparation is injected directly into the muscle to be treated. At physiological pH, the toxin is released from the protein complex and the desired pharmacological effect takes place. The effect of Botulinum toxin is only temporary, which is the reason why repeated administration of Botulinum toxin may be required to maintain a therapeutic affect.

[0005] The Clostridial Neurotoxins weaken voluntary muscle strength and are effective therapy for strabism, focal dystonia, including cervical dystonia, and benign essential blepharospasm. They have been further shown to relief hemifacial spasm, and focal spasticity, and moreover, to be effective in a wide range of other indications, such as gastrointestinal disorders, hyperhidrosis, and cosmetic wrinkle correction, see Jost 2007, Drugs 67, 669.

[0006] During the manufacturing process of Clostridial Neurotoxins, the qualtitative and quantitative determination as well as the quality control of the active Neurotoxin polypeptide is of particular importance. Currently available Neurotoxin preparations comprise, in addition to the desired active (processed or mature) Neurotoxin, a proteolytically unprocessed precursor and/or partially processed Neurotoxin polypeptide. The proteolytically unprocessed precursor or partially processed Neurotoxin polypeptide differs from the mature (active, processed) Neurotoxin polypeptide in a sequence of only a few amino acids. Therefore, they can hardly be quantitatively distinguished based on their chemical and physical properties. On the other hand, the portion of proteolytically unprocessed precursor and/or partially processed Neurotoxin polypeptide of the total

protein may still be significant in such preparations. The portion depends on the biological system used for the production and results from the biosynthesis and the conditions of the fermentation process. Thus, the amount of desired mature, biologically active Neurotoxin polypeptide in Neurotoxin preparations is predefined and, currently, rather difficult to determine.

[0007] Means and methods for a reliable qualitative and quantitative detection system of mature (active) Neurotoxin polypeptide are highly desirable but not yet available.

[0008] Thus, the technical problem underlying the present invention may be seen as the provision of means and methods complying with the aforementioned needs. The technical problem is solved by the embodiments characterized in the claims and herein below.

[0009] The present invention relates to a method for determining the amount of processed (active) Neurotoxin polypeptide in a solution comprising processed Neurotoxin polypeptide and partially processed and/or unprocessed Neurotoxin polypeptide comprising the steps of:

a) contacting a first portion of said solution with a first capture antibody which specifically binds to the light chains of mature Neurotoxin polypeptide, partially processed, and unprocessed Neurotoxin polypeptide under conditions which allow for binding of said antibody to said mature Neurotoxin, partially processed, and unprocessed Neurotoxin polypeptide, thus forming a first antibody-complex,

b) contacting the first antibody complex with a detection antibody which specifically binds to the heavy chain of said mature Neurotoxin, partially processed, and unprocessed Neurotoxin polypeptide in the antibody complex formed in step a), whereby a first detection complex is formed,

c) contacting a second portion of said solution with a second capture antibody which specifically binds to the linkers of said partially processed and unprocessed Neurotoxin polypeptide under conditions which allow for binding of said antibody to said partially processed and unprocessed Neurotoxin polypeptide, and thus forming a second antibody-complex,

d) contacting the second antibody-complex with the detection antibody, whereby a second detection complex is formed,

e) determining the amount of the second detection complex formed in steps b) and d), and

f) calculating the amount of mature Neurotoxin polypeptide based on the amounts of the first and second detection complex determined in step e).

[0009.1] The present invention also relates to an in vitro method for determining of the amount of a processed Clostridial Neurotoxin polypeptide in a solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, the method comprising the steps of:

a) contacting a first portion of said solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, with a first capture antibody which specifically binds to the light chain of processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said first capture antibody to said light chain of processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide, thus forming a first antibody complex,

b) contacting said first antibody complex with a detection antibody which specifically binds to the heavy chain of said processed, unprocessed and partially processed Clostridial Neurotoxin polypeptide in the antibody complex formed in step a), whereby a first detection complex is formed,

c) contacting a second portion of said solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, with a second capture antibody which specifically binds to a linker of said partially processed or unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said second capture antibody to said partially processed or unprocessed Clostridial Neurotoxin polypeptide, wherein said second capture antibody specifically binds to a peptide epitope consisting of an amino acid sequence as defined in any one of SEQ ID NOs: 1 to 16, thus forming a second antibody complex,

d) contacting said second antibody complex with a detection antibody which is different from the detection antibody in step b) and which specifically binds to the antibody complex formed in step c), whereby a second detection complex is formed,

e) determining the amount of the first detection complex formed in step b) and the second detection complex formed in step d), and

f) calculating the amount of processed Clostridial Neurotoxin polypeptide based on the amounts of the first and second detection complex determined in step e).

[0009.2] The present invention also relates to an in vitro method for the determination of the amount of processed Clostridial Neurotoxin polypeptide in a solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, the method comprising the steps of:

a) contacting a first portion of said solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, with a first capture antibody which specifically binds to the heavy chain of processed, partially processed, and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said first capture antibody to said heavy chain of processed, partially processed, and unprocessed Clostridial Neurotoxin polypeptide, thus forming a first antibody complex,

b) contacting the first antibody complex with a detection antibody which specifically binds to the light chain of said processed, partially processed, and unprocessed Clostridial Neurotoxin polypeptide in the antibody complex formed in step a), whereby a first detection complex is formed,

c) contacting a second portion of said solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, with a second capture antibody which specifically binds to a linker of said partially processed and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said antibody to said partially processed and unprocessed Clostridial Neurotoxin polypeptide, wherein said second capture antibody specifically binds to a peptide epitope consisting of an amino acid sequence as defined in any one of SEQ ID NOs: 1 to 16, thus forming a second antibody complex,

d) contacting said second antibody complex with a detection antibody which is different from the detection antibody in step b) and which specifically binds to the antibody complex formed in step c), whereby a second detection complex is formed,

e) determining the amount of the first detection complex formed in step b) and the second detection complex formed in step d), and

f) calculating the amount of processed Clostridial Neurotoxin polypeptide, based on the amounts of the first and second detection complex determined in step e).

[0009.3] The present invention also relates to a kit for determining of the amount of a processed Clostridial Neurotoxin polypeptide in a solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, the kit comprising:

a) a first capture antibody which specifically binds to the light chain of processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said first capture antibody to said processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide, thereby forming a first antibody complex;

b) a detection antibody which specifically binds to the heavy chain of said processed, unprocessed and partially processed Clostridial Neurotoxin polypeptide in the first antibody complex, whereby a first detection complex is formed;

c) a second capture antibody which specifically binds to a linker of said partially processed or unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said second capture antibody to said partially processed or unprocessed Clostridial Neurotoxin polypeptide, wherein said second capture antibody specifically binds to a peptide epitope consisting of an amino acid sequence as defined in any one of SEQ ID NOs: 1 to 16, thereby forming a second antibody complex;

d) a detection antibody which is different from the detection antibody in step b) and which specifically binds to the second antibody complex formed in step c), whereby a second detection complex is formed;

e) means for calculating the amount of processed Clostridial Neurotoxin polypeptide, based on the amounts of said first and second detection complex; and

f) instructions for carrying out the forming of a first antibody complex, the forming of a second antibody complex, the determining of the amounts of the first antibody complex and of the second antibody complex and the calculating of the amount of processed Clostridial Neurotoxin polypeptide.

[0009.4] The present invention also relates to a kit for determining of the amount of a processed Clostridial Neurotoxin polypeptide in a solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, the kit comprising:

a) a first capture antibody which specifically binds to the heavy chains of processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said first capture antibody to said processed,

partially processed and unprocessed Clostridial Neurotoxin polypeptide, thereby forming a first antibody complex;

b) a detection antibody which specifically binds to the light chains of said processed, unprocessed and partially processed Clostridial Neurotoxin polypeptides in the first antibody complex, whereby a first detection complex is formed;

c) a second capture antibody which specifically binds to a linker of said partially processed or unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said second capture antibody to said partially processed or unprocessed Clostridial Neurotoxin polypeptide, wherein said second capture antibody specifically binds to a peptide epitope consisting of an amino acid sequence as defined in any one of SEQ ID NOs: 1 to 16, thereby forming a second antibody complex;

d) a detection antibody which is different from the detection antibody in step b) and which specifically binds to the second antibody complex formed in step c), whereby a second detection complex is formed;

e) means for calculating the amount of processed Clostridial Neurotoxin polypeptide, based on the amounts of said first and second detection complex; and

f) instructions for carrying out the forming of a first antibody complex, the forming of a second antibody complex, the determining of the amounts of the first antibody complex and of the second antibody complex and the calculating of the amount of processed Clostridial Neurotoxin polypeptide.

[0010] The aforementioned method may, in general, comprise additional steps including steps for the preparation of the solution or steps concerning the further evaluation of the results obtained in step f). Moreover, the steps a) and b) as well as steps c) and d) may be carried out simultaneously or sequentially. In the latter case, steps a) and b) can be carried out prior or after steps c) and d). Further the determination referred to in step e) may be carried out in said case after both series of steps have been carried out or the determination in step e) as far as the first detection complex will be concerned is carried out after steps a) and b) while the determination concerning the second detection complex is carried out after steps c) and d). The method may in part or entirely be assisted by automation. The incubation and measurement steps can be carried out by, e.g., a robot. The data analysis and interpretation can be carried out by a computer-implemented calculation algorithm.

[0011] The term "Neurotoxin polypeptide" as used in the present invention refers to the seven distinct serotypes of Botulinum Neurotoxins, i.e. BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, and to Tetanus Neurotoxin (TeNT), see Table 1, and variants thereof.

Table 1: Botulinum and Tetanus Neurotoxins

SEQ ID NO:	Reference	Accession-NO:	Neurotoxin (full length)/ Bacterial Strain
17	Beecher 1997, J Protein Chem 16, 701-712.; Krieglstein 1994, J Protein Chem 13, 49-57.	ABD65472.1 GI:89258592	BoNT/A (Hall/62A)
18	Antharavally 1998, J Protein Chem 17, 417-428.	BAE48264.1 GI:81230332	BoNT/B (Okra)
19	Sagane 1999, J Protein Chem 18, 885-892.	BAA89713.1 GI:6729213	BoNT/C1 (C-6814)
20	Sagane 1999, J Protein Chem 18,885-892.	BAA90661.1 GI:6939795	BoNT/D (CB16)
21	Antharavally 1997, J Protein Chem 16, 787-799.	CAA43999.1 GI:40394	BoNT/E (Beluga)
22	Sagane 1999, J Protein Chem 18, 885-892.	CAA73972.1 GI:3805790	BoNT/F (NCTC10281)

SEQ ID NO:	Reference	Accession-NO:	Neurotoxin (full length)/ Bacterial Strain
23	Campbell 1993, Biochim. Biophys. Acta 1216 (3), 487-491	CAA52275.1 GI:441276	BoNT/G
24	Krieglstein 1991, Eur J Biochem 202, 41-51.; Krieglstein et al. 1990, Eur J Biochem 188, 39-45.	P04958.2 GI:135624	TeNT

[0012] The Neurotoxins referred to herein, in principle, comprise an N-terminal light chain and a C-terminal heavy chain. The Neurotoxins are produced as single chain precursor molecules, herein referred to as "unprocessed Neurotoxin polypeptides". The N-terminal light chain and the C-terminal heavy chain sequences are separated in the unprocessed Neurotoxins by at least one proteolytic cleavage site. These Neurotoxins contain a linker sequence between the light and heavy chain sequences, wherein the light chain is located N-terminally starting from the first cleavage site and the heavy chain is located C-terminally starting from the second cleavage site. In an aspect of the invention, said linker has an amino acid sequence as shown in any one of SEQ ID NOs: 1 to 16. During processing of the Neurotoxins, the linker sequence will be excised. These Neurotoxins contain two proteolytic cleavage sites, one at the N-terminal and one at the C-terminal end of the linker sequences. During processing of such Neurotoxins, intermediates may occur which are cleaved on either cleavage site, i.e. the linker sequence will not be yet excised but remains on either the N-terminal light chain or the C-terminal heavy chain. Such intermediates are referred to as "partially processed Neurotoxin polypeptides" in this specification. Other Neurotoxins, merely, contain one cleavage site. For those Neutrotoxins it will be understood that no linker sequence can be excised. Nevertheless, the unprocessed Neurotoxin can be immunologically recognized by an intact proteolytic cleavage site and flanking sequences. These flanking sequences and the cleavage site are also deemed to be a linker for the purpose of the present invention. Thus, the term "linker" as used herein and specified above refers either to the sequence between the light and heavy chain sequences for Neurotoxin polypeptides having two cleavage sites or to the cleavage site and flanking sequences for Neurotoxin polypeptides having only a single cleavage site. As a result of the processing, "processed Neurotoxin polypeptide" is obtained. The said processed Neurotoxin polypeptide exhibits the biological properties characteristic for a Neurotoxin, namely, (a) receptor binding, (b) internalization, (c) translocation across the endosomal membrane into the cytosol, and/or (d) endoproteolytic cleavage of proteins involved in synaptic vesicle membrane fusion. Therefore, the processed Neurotoxin polypeptide is

sometimes referred to herein as active or mature Neurotoxin polypeptide. The biological activity of the Neurotoxin polypeptides, in an aspect, results from all of the aforementioned biological properties. In vivo assays for assessing biological activity include the mouse LD50 assay and the ex vivo mouse hemidiaphragm assay as described by Pearce et al. and Dressier et al. (Pearce 1994, Toxicol Appl Pharmacol 128: 69-77 and Dressier 2005, Mov Disord 20:1617-1619). The biological activity is commonly expressed in Mouse Units (MU). As used herein, 1 MU is the amount of neurotoxic component, which kills 50% of a specified mouse population after intraperitoneal injection, i.e. the mouse i.p. LD50.

[0013] In an aspect of the method of the invention, the said Neurotoxin polypeptide is selected from the group consisting of: a) a Neurotoxin polypeptide having an amino acid sequence as shown in any one of SEQ ID NOs: 17 to 24, and b) a Neurotoxin polypeptide having an amino acid sequence being at least 40% identical to the amino acid sequence of the Neurotoxin polypeptide as shown in any one of SEQ ID NOs: 17 to 24. The aforementioned amino acid sequences show unprocessed Neurotoxin polypeptides. The sequences of the corresponding partially processed or processed Neurotoxin polypeptides can be deduced from the said sequences by the information on cleavage sites provided in Table 3, below. In another aspect of the invention, the Neurotoxin polypeptide has an amino acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% sequence identical to the amino acid sequence as shown in SEQ ID NOs: 17 to 24. Identical as used in the present invention refers to sequence identity of amino acid sequences wherein the sequences are aligned so that the highest order match is obtained. This can be achieved by using published techniques or methods codified in computer programs such as, for example, BLASTP, BLASTN, FASTA, Altschul 1990, J Mol Biol 215, 403. The percent identity values are, in one aspect, calculated over the entire amino acid sequence. A series of programs based on a variety of algorithms is available to the skilled worker for comparing different sequences. In this context, the algorithms of Needleman and Wunsch or Smith and Waterman give particularly reliable results. To carry out the sequence alignments, the program PileUp (1987, J Mol Evolution 25, 351; Higgins 1989 CABIOS 5, 151) or the programs Gap and BestFit (Needleman and Wunsch 1970, J Mol Biol 48; 443; Smith and Waterman 1981, Adv Appl Math 2, 482), which are part of the GCG software packet (Genetics Computer Group 1991, 575 Science Drive, Madison, Wisconsin, USA 53711), are to be used. The sequence identity values recited above in percent (%) are to be determined, in one aspect of the invention, using the program GAP over the entire sequence region with the following settings: Gap Weight: 50, Length Weight: 3, Average Match: 10.000 and Average Mismatch: 0.000, which, unless otherwise

specified, shall always be used as standard settings for sequence alignments. It will be understood that the aforementioned variants shall, in an aspect of the invention, retain, at least one of the biological properties of Neurotoxins and, in an aspect, all of the the biological properties of a Neurotoxin polypeptide recited herein. In a further aspect, the variants can be Neurotoxins having improved or altered biological properties, e.g., they may comprise cleavage sites which are improved for enzyme recognition or may be improved for receptor binding or any other property specified above. It is conceivable that the concept of the present invention relies on the presence of two or more cleavage sites between light and heavy chain of the Neurotoxin polypeptide while the nature of the cleavage sites and the particular amino acid sequence between them does not matter as long as the agent is specific for the partially processed or unprocessed Neurotoxin polypeptide. Accordingly, it is another aspect, to replace protease recognition sites and the linker peptide between heavy- and light chain of the Neurotoxin polypeptide.

[0014] In another aspect, the Neurotoxin polypeptide in accordance with the method of the invention may be a chimeric molecule. Such said chimeric molecule, in one aspect, may have single domains substituted. Accordingly, in another aspect, the portion of the Neurotoxin heavy chain is replaced by a portion of an FC domain of an antibody.

[0015] The term "amount" as used in the method of the present invention encompasses the absolute amount of a polypeptide, the relative amount or the concentration of the said polypeptide as well as any value or parameter which correlates thereto or can be derived therefrom.

[0016] The term "solution" as used herein refers to any solvent system containing mature Neurotoxin polypeptide and its partially processed and/or unprocessed Neurotoxin polypeptide precursors. The solvent system furthermore comprises a solvent. The solvents encompassed, in various aspects of the invention, are water, aqueous buffer systems, organic solvents, and ionic liquids. In one aspect of the invention, it is an aqueous solvent system. Moreover, the solvent system, in addition to the mature Neurotoxin polypeptide and the partially processed or unprocessed precursor Neurotoxin polypeptide and the solvent may comprise further molecules as well, including further bacterial polypeptides. In an aspect, the solution to be applied in the method of the present invention will be a bacterial cell culture or a partially purified or purified preparation obtained from such a bacterial cell culture.

[0017] The term "portion" as used in accordance with the method of the invention, refers to a sample or aliquot of the solution. In an aspect of the method of the invention, the first portion and the second portion referred to in this invention are essentially equal in their volume and contents. This can be achieved, e.g., by measuring the total protein content of the first and second portion, whereby an essentially identical total protein content is indicative for a first and second portion having essentially the same contents. However, in a further aspect, a portion to be applied as a first or second portion may be a dilution of the sample or aliquot of the solution. It will be understood that dependent on the amount of the Neurotoxin polypeptide to be determined (i.e. partially processed or unprocessed Neurotoxin polypeptide or total Neurotoxin), a dilution might become necessary in order to allow for an optimal qualitative and quantitative determination. How to make such dilutions is well known to those skilled in the art.

[0018] The term "contacting" as used in accordance with the method of the invention refers to (i) bringing the aforementioned capture antibodies and the Neurotoxins comprised by the solution or (ii) bringing the antibody-complexes and the detection antibodies in physical proximity as to allow physical and/or chemical interaction. Suitable conditions which allow for specific interaction are well known to the skilled worker. Said conditions will depend on the antibodies and the solution to be applied in the method of the present invention and can be adapted by the skilled artisan without further ado. Moreover, a time being sufficient to allow interaction can also be determined by the skilled worker without further ado. Moreover, it is to be understood that between the individual steps of contacting recited in the method of the present invention, washing steps may be performed in order to obtain suitable conditions for contacting. For example, after formation of a first antibody-complex in step a), the remaining solution shall be removed prior to applying the detection antibody to the said antibody-complex. Furthermore, after the first detection-complex is formed in step b), it might be necessary to remove the remaining (uncomplexed) detection antibody prior to determining the amount of the first detection-complex in step c). The same applies, of course, for steps d) to f), accordingly.

[0019] An "antibody" as used herein encompasses a monoclonal antibody, a polyclonal antibody, a single chain antibody, a chimerized antibody, a bispecific antibody, a synthetic antibody, or a fragment of any of said antibodies. Fragments of said antibodies include Fab, Fv , or scFv fragments, or chemically modified derivatives of any of these fragments. Antibodies can be manufactured by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. Monoclonal antibodies can be prepared by the techniques originally described in Köhler

1975, Nature 256, 495, and Galfré 1981, Meth Enzymol 73, 3. Said techniques comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Antibodies can be further improved by techniques well known in the art. For example, surface plasmon resonance as employed in the BIACORE(R) system can be used to increase the efficiency of phage antibodies which bind to the epitope, see Schier 1996, Human Antibodies Hybridomas 7, 97; Malmborg 1995, J. Immunol Methods 183, 7. Antibodies as used herein also comprise functional equivalents of antibodies, i.e. agents which are capable of specifically binding to the desired epitopes or parts of the Neurotoxin polypeptides. In an aspect, such functional equivalents comprise the receptor or binding proteins referred to elsewhere in this specification or domains thereof which are capable of mediating the said specific binding.

[0020] According to the method of the present invention, the "first capture antibody" specifically binds to epitopes comprised by the light chain of mature Neurotoxin polypeptide and comprised by the partially processed and/or unprocessed Neurotoxin polypeptide. Specific binding as used herein, in general, means that the antibody does not cross react to a significant extent with other epitopes on the heavy chain or the linker of the Neurotoxin polypeptide to be determined or on other polypeptides. Specific binding as referred to herein can be tested by various well known techniques including, e.g., competition experiments and Western blots. An epitope as used in accordance with the invention relates to the antigenic determinant which is recognized by the antibody.

[0021] In another aspect the, different capture antibodies can be used to replace the first capture antibody. To this end, at least one capture antibody specifically binds to epitopes of the light chain of the unprocessed Neurotoxin polypeptide, at least one further capture antibody specifically binds to epitopes of the light chain of the partially processed Neurotoxin polypeptide and at least one further capture antibody specifically binds to epitopes of the light chain of the processed Neurotoxin polypeptide may be applied. It will be understood that these three types of antibodies functionally resemble the first capture antibody for the purpose of the method of the present invention. Similarly, a capture antibody which specifically binds to epitopes of the light chain of partially processed and unprocessed Neurotoxin polypeptide can be used in combination with a capture antibody specifically binds to epitopes of the light chain of the processed Neurotoxin polypeptide.

[0022] The said first capture antibody shall, in an aspect, be immobilized. Said immobilization of an antibody, in principle, can be achieved, in an aspect, by reversible or non-reversible, direct or indirect (via linker molecules) binding of the antibody to a solid

support. In an aspect the first capture antibody is immobilized prior to carrying out the method. In another aspect, the first capture antibody is immobilized after the first antibody complex has been formed but prior to contacting the complex with the detection antibody. Materials for solid supports are well known in the art and include, inter alia, commercially available polysaccharide matrices selected from the group consisting of: sepharose, sephadex; agarose, sephacell, micro-cellulose, and alginate-beads, polypeptide matrices, polystyrene beads, latex beads, magnetic beads, colloid metal particles, glass, plastic and/or silicon chips and surfaces, nitrocellulose strips, membranes, sheets, duracytes, wells and walls of reaction trays, plastic tubes. In an aspect of the invention, said solid support is made of gamma-irradiated polysterene.

[0023] The term "first antibody-complex" refers to a complex comprising the first capture antibody specifically bound to the processed, partially processed, or unprocessed Neurotoxin polypeptides. The said antibody-complex is formed as the result of contacting the first capture antibody with the solution comprising the said processed, partially processed and/or unprocessed Neurotoxin polypeptides as set forth above.

[0024] According to the method of the invention, the "second capture antibody" specifically binds to an epitope which comprises the linker of unprocessed and/or partially processed Neurotoxin polypeptide or parts thereof. In cases where a linker sequence is missing, it is envisaged that the said second capture antibody specifically binds to an epitope comprising the uncleaved proteolytic cleavage site or parts thereof. In an aspect of the invention, the second capture antibody does not cross react with the processed Neurotoxin polypeptide to a significant extent. In an aspect, said second immobilized capture antibody specifically binds to an epitope essentially consisting of, comprising or being comprised by an amino acid sequence as shown in SEQ ID NO: 1 to 16, see Table 2 or 3 below.

Table 2: Amino acid sequences of the cleavage sites of different Neurotoxin polypeptides and flanking sequences

SEQ ID NO:	Sequence of the epitope including cleavage sites (highlighted)	Neurotoxin (Bacterial Strain)
1	KLLCVRGIITSK**TKSLDKGYNK**ALN....DLCIKV	BoNT/A (Hall/62A)
2	IQMCKSV**K**APG.....ICIDV	BoNT/B (Okra)
3	TKFCHKAIDGR**SL....YNK**TL.....DCRELLV	BoNT/C1 (C-6814)

SEQ ID NO:	Sequence of the epitope including cleavage sites (highlighted)	Neurotoxin (Bacterial Strain)
4	TKVCLRLTK.....**NSR**D.....DSTCIKV	BoNT/D
5	IRFCKNIVSVK**G**.....**IR**K.....SICIEI	BoNT/E (Beluga)
6	VKFCKSVIPR**KG**.....**TK**AP.....PRLCIRV	BoNT/F (NCTC10281)
7	IAMCKPVMYKNT.....GKS.....EQCIIV	BoNT/G
8	IGLCKKIIPPTNI**R**ENLYN**R**TASLTDLGGELCIKI	TeNT

Table 3: Amino acid sequences of the linker regions

SEQ ID NO:	Sequence of the epitopes	Cleavage sites	Neurotoxin/ Bacterial Strain
9	TKSLDKGYNK	K438 / T439 K448 / A449	BoNT/A (Hall/62A)
10	CKSVKAPGIC	K441 / A442	BoNT/B (Okra)
11	SLYNK	R444 / S445 K449 / T450	BoNT/C1 (C-6814)
12	NSR	K442 / N443 R445 / D446	BoNT/D (CB16)
13	GIR	K419 / G420 R422 / K423	BoNT/E (Beluga)
14	KGTK	R435 / K436 K439 / A440	BoNT/F (NCTC10281)
15	NGTK		BoNT/G
16	ENLYNR	R449 (alternatively R455)	TeNT

[0025] Due to the presence of the aforementioned epitope, the unprocessed or partially processed Neurotoxin polypeptides can be specifically bound by the second capture antibody, and, thus, form a second antibody-complex. The said second capture antibody is, in an aspect, immobilized as explained in detail above.

[0026] Accordingly, the term "second antibody-complex" refers to a complex comprising the second capture antibody specifically bound to partially processed or unprocessed Neurotoxin polypeptide. The said second antibody-complex, however, shall not comprise processed Neurotoxin polypeptide.

[0027] According to the method of the invention, the "detection antibody" specifically binds to the first and/or second antibody-complex. In an aspect, the detection antibody for the first and the second antibody-complex is identical. However, in a further aspect, different detection antibodies may be used for the first and the second antibody-complex. In an aspect, the detection antibody specifically binds to epitopes on the heavy chain of the processed, partially processed and unprocessed Neurotoxin polypeptide. Due to the presence of the same epitope in both complexes, the first antibody-complex or the second antibody-complex can be specifically bound and, thus, be detected by the detection antibody in said aspect of the invention.

[0028] As a result of the specific binding of the detection antibody, a first detection complex or a second detection complex is formed, respectively.

[0029] Therefore, the term "first detection complex" refers to a complex comprising the first antibody-complex and the detection antibody. Likewise, the term "second detection complex" refers to a complex comprising the second antibody-complex and the detection antibody.

[0030] In an aspect of the method of the invention, said detection antibody comprised by the first or second detection complex is coupled to a detectable label allowing the measurement of the amount of the detection antibody which is bound to the detection complex. By measuring the said amount of bound detection antibody, the amount of first or second antibody-complexes can be determined since the amount of bound detection antibody in the detection complex correlates with the amount of antibody-complex comprised by the detection complex. Labeling may be done by direct or indirect methods. Direct labeling involves binding of the label directly (covalently or non-covalently) to the first detection antibody. Indirect labeling involves binding (covalently or non-covalently) of an agent which specifically binds to the detection antibody and which carries a detectable label. Such an agent may be, e.g., a secondary (higher order) antibody which specifically binds to the detection antibody. The secondary antibody in such a case will be coupled to a detectable label. It will be understood that further higher order antibodies can be used in addition for detection of the detection complex. The higher order antibodies are often used to increase the signal. Suitable higher order antibodies may also include the well-known streptavidin-biotin system (Vector Laboratories, Inc.), and the well-known Dako LSAB™2 and LSAB™+ (labeled streptavidin-biotin), or Dako PAP (Peroxidase Anti-Peroxidase). In a further aspect, the said label of the first detection antibody is

selected from the group consisting of: fluorescent dyes, chemoluminescent molecules, radioactive labels and enzymes capable of generating a detectable signal. Typical fluorescent labels include fluorescent proteins (such as GFP and its derivatives), Cy3, Cy5, Texas Red, Fluorescein, and the Alexa dyes (e.g. Alexa 568). Typical radioactive labels include ^{35}S, ^{125}I, ^{32}P, ^{33}P and the like. Alternatively, a detectable label coupled to the said first detection antibody may also be an enzyme which is capable of generating a detectable signal, e.g., by conversion of a substrate. In an aspect, such an enzyme may be a peroxidase (e.g., horseradish peroxidase) or alkaline phosphatase.

[0031] The term "determining the amount" as used herein relates to measuring the absolute amount, relative amount or concentration in a quantitative or semi-quantitative manner. Measuring will be done based on the chemical, physical or biological properties of the detectable label coupled to the first detection antibody. Suitable measures for detection are well known to those skilled in the art and depend on the nature of the detectable label as set forth above. It will be understood, however, that the amount of detectable label which can be measured correlates directly to the amount of detection complex which again correlates to the amount of antibody complex and, thus, to the amount of the Neurotoxin species to be determined, i.e. to either the total (processed, unprocessed and partially processed Neurotoxin) or the unprocessed and partially processed Neurotoxin. It will be understood that the determination of the amount of Neurotoxin polypeptides, in an aspect, also requires calibration of the method by applying standard solutions with predefined amounts of Neurotoxin polypeptides. How to carry out such a calibration is well known to those skilled in the art.

[0032] The term „calculating" as used in accordance with the method of the present invention relates to mathematical operations which allow for determining the amount of processed Neurotoxin based on the amounts of total Neurotoxin (i.e. processed, unprocessed and partially processed Neurotoxin) and the amount of partially processed and unprocessed Neurotoxin. In an aspect of the method of the present invention, said calculating includes subtraction of the amount of partially processed and unprocessed Neurotoxin from the amount of total Neurotoxin.

[0033] Advantageously, the method of the present invention allows for a reliable determination of the amount of processed Neurotoxin in a given preparation. Accordingly, the quality of Neurotoxin preparations can be increased since the preparations can be tested for constant amounts of the desired processed Neurotoxin polypeptide.

[0034] In principle, the method of the present invention can be carried out by coupling a first capture antibody to a solid support such as a reaction vial. Similarly, the second capture antibody shall be coupled to another physically separate solid support (e.g., a further reaction vial). Both capture antibodies coupled to the solid supports will subsequently be brought into contact to the said portions of the solution comprising the processed, unprocessed and/or partially processed Neurotoxin to be determined. Such a solution could be, e.g., a purified bacterial cell culture from Clostridum sp. It will be understood that a first portion will be brought into contact with the first capture antibody on the first solid support and the second portion will be brought into contact with the second capture antibody on the second solid support. The portions are usually of equal volume and are normalized with respect to their contents, e.g., their total protein content. Contacting will be carried out for a time sufficient to allow specific binding of the first and second capture antibodies to their respective antigens. For example, contacting can be carried out at room temperature for approx. an hour. Subsequently, the first and second portion of the solution will be discarded and the solid supports (e.g., reaction vials) will be washed once or twice by a buffer under conditions which do not affect the first and second antibody-complexes which have been meanwhile formed with the capture antibodies on the solid supports. After the washing steps have been carried out, the (first) detection antibody will be added to the solid supports under conditions which allow for specific binding of the detection antibody. Excess detection antibody shall be removed by further washing steps using an appropriate buffer. Subsequently, the amount of the first and the second detection complex can be determined by determination of the amount of specifically bound detection antibody. This will be achieved dependent on the nature of the label of the detection antibody, e.g., by measuring the optical density or the intensity of fluorescence. The measured amount for the detectable label can be compared with calibration standards in order to determine the amount of a Neurotoxin species, i.e. either the total (processed, unprocessed and partially processed Neurotoxin) or the unprocessed and partially processed Neurotoxin in the first or second detection complex. It will be understood that the first detection complex represents the amount of total Neurotoxin while the second detection complex represents the amount of partially processed and unprocessed Neurotoxin polypeptides, only. Accordingly, the amount of processed Neurotoxin polypeptide can be calculated in the aforementioned setup by subtracting the amount of the partially processed or unprocessed Neurotoxin polypeptide from the total Neurotoxin polypeptide amount.

[0035] It is to be understood that the definitions and explanations of the terms made above apply mutatis mutandis for all aspects described in this specification in the following except as otherwise indicated.

[0036] The present invention also relates to a method for the determination of the amount of processed (active) Neurotoxin polypeptide in a solution comprising processed Neurotoxin polypeptide and partially processed and/or unprocessed Neurotoxin polypeptide comprising the steps of:

a) contacting a first portion of said solution with a first capture antibody which specifically binds to the heavy chains of mature Neurotoxin polypeptide, partially processed, and unprocessed Neurotoxin polypeptide under conditions which allow for binding of said antibody to said mature Neurotoxin, partially processed, and unprocessed Neurotoxin polypeptide, thus forming a first antibody-complex,

b) contacting the first antibody complex with a detection antibody which specifically binds to the light chain of said mature Neurotoxin, partially processed, and unprocessed Neurotoxin polypeptide in the antibody complex formed in step a), whereby a first detection complex is formed,

c) contacting a second portion of said solution with a second capture antibody which specifically binds to the linkers of said partially processed and unprocessed Neurotoxin polypeptide under conditions which allow for binding of said antibody to said partially processed and unprocessed Neurotoxin polypeptide, and thus forming a second antibody-complex,

d) contacting the second antibody-complex with the detection antibody , whereby a second detection complex is formed,

e) determining the amount of the second detection complex formed in step b) and step e), and

f) calculating the amount of mature Neurotoxin polypeptide based on the amounts of the first and second detection complex determined in step e).

[0037] In another aspect of the methods of the invention, said methods further comprises determining the binding activity of Neurotoxin polypeptide.

[0038] The term "binding activity" as used in accordance with the method of the invention relates to the capability of the processed Neurotoxin polypeptide to a surface receptor protein which is present, e.g., on peripheral cholinergic nerve endings. Receptor proteins include in aspect SV2 for BoNT/A, synaptotagmins I and II for BoNT/B and BoNT/G, and a ganglioside (GT_{1B}) coreceptor. In an aspect of the method of the invention, said binding

activity can be determined ex vivo using a model substrate which substitutes the surface protein receptor by mimicking its binding domain. Said model substrate is, in an aspect, a labeled peptide derived from the aforementioned receptor proteins. In a further aspect, suitable labels include those mentioned elsewhere in this specification, and, in particular, biotin.

[0039] Thus, the present invention also contemplates a method for determining the binding activity of a Neurotoxin polypeptide comprising the steps of

a) contacting a portion of a Neurotoxin polypeptide containing solution with a labeled peptide, whereby a complex is formed, and
b) determining the said complex formed in step (a) based on the label, whereby the presence or absence of the complex or its amount is indicative for the binding activity of the Neurotoxin polypeptide in said solution.

[0040] The complex can be determined based on the nature of the label which has been used to label the peptide. In an aspect, e.g., the biotinylated peptide comprised by a complex can be determined by a Streptavidin conjugate capable of generating a detectable signal. The presence, absence or intensity will be indicative for the binding activity of the Neurotoxin polypeptides in the solution or its strength.

[0041] In another aspect of the method of the invention, said method further comprises determining the proteolytic activity of Neurotoxin polypeptide.

[0042] The term "proteolytic activity" as used in accordance with the method of the invention relates to the capability of processed Neurotoxin to proteolytically cleave N-ethylmaleimide-sensitive attachment receptor (SNARE) proteins involved in synaptic vesicle membrane fusion. In an aspect, said cleavage is zinc(II)-dependent. The said proteolytic activity can be determined using a model substrate which substitutes a naturally occurring SNARE protein. Moreover, upon cleavage, a detectable label such as a dye shall be released from the said model substrate. In one aspect, the model substrate is a compound having the general formula X-para-Nitroanilid, wherein X is Arginine or peptide having the sequence Arginine-Y, wherein Y represents one or more amino acids, and in another aspect, the compound is Arginine-para-Nitroanilid.

[0043] Thus, the present invention further contemplates a method for determining the proteolytic activity of a Neurotoxin comprising the steps of

a) contacting a portion of a Neurotoxin polypeptide containing solution with a compound having the general formula: X-para-Nitroanilide, wherein X is Arginine or a peptide having the sequence Arginine-Y, wherein Y represents one or more amino acids, and

b) determining the proteolytic activity of Neurotoxin polypeptide in said solution based on the amount of released para-Nitroaniline from step b) which correlates to the amount of Neurotoxin polypeptide.

[0044] In an aspect, Y represents a peptide residue having an amino acid sequence as shown in any one of SEQ ID NOs: 25 or 26.

[0045] The processed Neurotoxin polypeptide comprised by the said portion of the solution can cleave and, thus, release para-Nitroaniline from the remaining peptide. Para-Nitroaniline is a dye well known in the art. Determining the proteolytic activity of Neurotoxin polypeptide in said solution is based on the amount of released para-Nitroaniline which correlates to the amount of Neurotoxin polypeptide.

[0046] The present invention also contemplates a device for determining the amount of processed Neurotoxin polypeptide in a solution comprising:

a) an arrangement of a first capture antibody, a second capture antibody and a detection antibody, wherein said arrangement allows for carrying out the steps a) to e) of the aforementioned methods; and

b) means for calculating the amount of mature Neurotoxin polypeptide based on the amounts of the first and second detection complex determined by the arrangement according to a).

[0047] The term "device" as used herein relates to a system comprising at least the aforementioned arrangement and means operatively linked to each other as to allow the determination. In an aspect, the arrangement can be a solid support with immobilized capture antibodies as referred to above which may be present in physically separate vials in order to allow a separate contacting with the first and second portion of the solution. Moreover, the device may comprise, in an aspect, a unit for the determination of the amount of the detection complexes. Dependent on the kind of detection antibody to be used, such a unit will comprise a detector for the signals generated by the detection antibody. Moreover, the unit can also comprise, in an aspect, means for calibration, e.g., a computer based algorithm, for comparing the measured signals to the calibration standards in order to determine the amounts of the Neurotoxin polypeptides present in a solution or

portion thereof. The device will also comprise means for calculating the amount of mature Neurotoxin polypeptide based on the amounts of the first and second detection complex, e.g., a computer-based algorithm for carrying out the calculation.

[0048] Further, the invention relates to a kit adapted for carrying out the aforementioned methods, said kit comprising:

a) an arrangement of a first capture antibody, a second capture antibody and a detection antibody, wherein said arrangement allows for carrying out the steps a) to e) of the aforementioned methods;

b) means for calculating the amount of mature Neurotoxin polypeptide based on the amounts of the first and second detection complex determined by the arrangement according to a); and

c) instructions for carrying out said method.

[0049] The term "kit" as used herein refers to a collection of the aforementioned means or reagents of the present invention which may or may not be packaged together. The components of the kit may be comprised by separate vials (i.e. as a kit of separate parts) or provided in a single vial. Moreover, it is to be understood that the kit of the present invention is to be used for practicing the methods referred to herein above. In one aspect, it is envisaged that all components are provided in a ready-to-use manner for practicing the methods referred to above. In a further aspect, the kit contains instructions for carrying out the said methods. The instructions can be provided by a user manual in paper- or electronic form. For example, the manual may comprise instructions for interpreting the results obtained when carrying out the aforementioned methods using the kit of the present invention.

Figures:

Figure 1: Scheme of the binding of at least one (or more) detection antibody. L: light chain; H: heavy chain; HRP: horseradish peroxidase.

Figure 2: Scheme of the specific binding of the second capture antibody to the partially processed or unprocessed precursor Neurotoxin polypeptide and the subsequent binding of at least one (or more) detection antibody. L: light chain; H: heavy chain; HRP: horseradish peroxidase.

Figure 3: Scheme of the determination of the binding activity of the Neurotoxin polypeptide. L: light chain; H: heavy chain; HRP: horseradish peroxidase; TMB: tetramethylbenzidine; red: reduced; ox: oxidized.

5

Figure 4: Scheme of the determination of the proteolytic activity of the Neurotoxin polypeptide. L: light chain; H: heavy chain.

Claims

1. An in vitro method for determining of the amount of a processed Clostridial Neurotoxin polypeptide in a solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, the method comprising the steps of:

a) contacting a first portion of said solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, with a first capture antibody which specifically binds to the light chain of processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said first capture antibody to said light chain of processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide, thus forming a first antibody complex,

b) contacting said first antibody complex with a detection antibody which specifically binds to the heavy chain of said processed, unprocessed and partially processed Clostridial Neurotoxin polypeptide in the antibody complex formed in step a), whereby a first detection complex is formed,

c) contacting a second portion of said solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, with a second capture antibody which specifically binds to a linker of said partially processed or unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said second capture antibody to said partially processed or unprocessed Clostridial Neurotoxin polypeptide, wherein said second capture antibody specifically binds to a peptide epitope consisting of an amino acid sequence as defined in any one of SEQ ID NOs: 1 to 16, thus forming a second antibody complex,

d) contacting said second antibody complex with a detection antibody which is different from the detection antibody in step b) and which specifically binds to the antibody complex formed in step c), whereby a second detection complex is formed,

e) determining the amount of the first detection complex formed in step b) and the second detection complex formed in step d), and

f) calculating the amount of processed Clostridial Neurotoxin polypeptide based on the amounts of the first and second detection complex determined in step e).

2. An in vitro method for the determination of the amount of processed Clostridial Neurotoxin polypeptide in a solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, the method comprising the steps of:

a) contacting a first portion of said solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, with a first capture antibody which specifically binds to the heavy chain of processed, partially processed, and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said first capture antibody to said heavy chain of processed, partially processed, and unprocessed Clostridial Neurotoxin polypeptide, thus forming a first antibody complex,

b) contacting the first antibody complex with a detection antibody which specifically binds to the light chain of said processed, partially processed, and unprocessed Clostridial Neurotoxin polypeptide in the antibody complex formed in step a), whereby a first detection complex is formed,

c) contacting a second portion of said solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, with a second capture antibody which specifically binds to a linker of said partially processed and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said antibody to said partially processed and unprocessed Clostridial Neurotoxin polypeptide, wherein said second capture antibody specifically binds to a peptide epitope consisting of an amino acid sequence as defined in any one of SEQ ID NOs: 1 to 16, thus forming a second antibody complex,

d) contacting said second antibody complex with a detection antibody which is different from the detection antibody in step b) and which specifically binds to the antibody complex formed in step c), whereby a second detection complex is formed,

e) determining the amount of the first detection complex formed in step b) and the second detection complex formed in step d), and

f) calculating the amount of processed Clostridial Neurotoxin polypeptide, based on the amounts of the first and second detection complex determined in step e).

3. The method of claim 1 or 2, wherein said first capture antibody is immobilized.

4. The method of any one of claims 1 to 3, wherein said second capture antibody is immobilized.

5. The method of any one of claims 1 to 4, wherein calculating in step f) comprises subtracting the determined amount of the second detection complex from the determined amount of the first detection complex.

6. The method of any one of claims 1 to 5, wherein said unprocessed Clostridial Neurotoxin polypeptide is:

a) a Clostridial Neurotoxin polypeptide as defined in any one of SEQ ID NOs: 17 to 24; or

b) a Clostridial Neurotoxin polypeptide having an amino acid sequence being at least 40% identical to the amino acid sequence of the Clostridial Neurotoxin polypeptide of a).

7. The method of any one of claims 1 to 6, wherein said method further comprises determining the binding activity of the processed Clostridial Neurotoxin polypeptide to a surface receptor protein.

8. The method of claim 7, wherein determining the binding activity comprises the steps of

i) contacting a portion of the Clostridial Neurotoxin polypeptide containing solution with a labeled peptide mimicking the binding domain of said surface receptor protein, whereby a complex is formed, and

ii) determining the said complex formed in step i) based on the label, whereby the presence or absence of the complex or its amount is indicative for the binding activity of the processed Clostridial Neurotoxin polypeptide in said solution.

9. The method of any one of claims 1 to 8, wherein said method further comprises determining the proteolytic activity of the processed Clostridial Neurotoxin polypeptide.

10. The method of claim 9, wherein determining the proteolytic activity comprises the steps of

iii) contacting a portion of the Clostridial Neurotoxin polypeptide containing solution with a compound having the general formula: X-para-Nitroanilide,

wherein X is Arginine or a peptide having the sequence Arginine-Y, wherein Y represents one or more amino acids, and

iv) determining the proteolytic activity of the processed Clostridial Neurotoxin polypeptide in said solution based on the amount of released para-Nitroaniline from step iii) which correlates to the amount of the processed Clostridial Neurotoxin polypeptide.

11. A kit for determining of the amount of a processed Clostridial Neurotoxin polypeptide in a solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, the kit comprising:

a) a first capture antibody which specifically binds to the light chain of processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said first capture antibody to said processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide, thereby forming a first antibody complex;

b) a detection antibody which specifically binds to the heavy chain of said processed, unprocessed and partially processed Clostridial Neurotoxin polypeptide in the first antibody complex, whereby a first detection complex is formed;

c) a second capture antibody which specifically binds to a linker of said partially processed or unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said second capture antibody to said partially processed or unprocessed Clostridial Neurotoxin polypeptide, wherein said second capture antibody specifically binds to a peptide epitope consisting of an amino acid sequence as defined in any one of SEQ ID NOs: 1 to 16, thereby forming a second antibody complex;

d) a detection antibody which is different from the detection antibody in step b) and which specifically binds to the second antibody complex formed in step c), whereby a second detection complex is formed;

e) means for calculating the amount of processed Clostridial Neurotoxin polypeptide, based on the amounts of said first and second detection complex; and

f) instructions for carrying out the forming of a first antibody complex, the forming of a second antibody complex, the determining of the amounts of the first antibody

complex and of the second antibody complex and the calculating of the amount of processed Clostridial Neurotoxin polypeptide.

12. A kit for determining of the amount of a processed Clostridial Neurotoxin polypeptide in a solution comprising processed Clostridial Neurotoxin polypeptide and partially processed and/or unprocessed Clostridial Neurotoxin polypeptide, the kit comprising:

a) a first capture antibody which specifically binds to the heavy chains of processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said first capture antibody to said processed, partially processed and unprocessed Clostridial Neurotoxin polypeptide, thereby forming a first antibody complex;

b) a detection antibody which specifically binds to the light chains of said processed, unprocessed and partially processed Clostridial Neurotoxin polypeptides in the first antibody complex, whereby a first detection complex is formed;

c) a second capture antibody which specifically binds to a linker of said partially processed or unprocessed Clostridial Neurotoxin polypeptide under conditions which allow for binding of said second capture antibody to said partially processed or unprocessed Clostridial Neurotoxin polypeptide, wherein said second capture antibody specifically binds to a peptide epitope consisting of an amino acid sequence as defined in any one of SEQ ID NOs: 1 to 16, thereby forming a second antibody complex;

d) a detection antibody which is different from the detection antibody in step b) and which specifically binds to the second antibody complex formed in step c), whereby a second detection complex is formed; and

e) means for calculating the amount of processed Clostridial Neurotoxin polypeptide, based on the amounts of said first and second detection complex; and

f) instructions for carrying out the forming of a first antibody complex, the forming of a second antibody complex, the determining of the amounts of the first antibody complex and of the second antibody complex and the calculating of the amount of processed Clostridial Neurotoxin polypeptide.

13. The kit of claim 11 or 12, wherein said unprocessed Clostdridial Neurotoxin polypeptide is:

a) a Clostridial Neurotoxin polypeptide as defined in any one of SEQ ID NOs: 17 to 24; or

b) a Clostridial Neurotoxin polypeptide having an amino acid sequence being at least 40% identical to the amino acid sequence of the Clostridial Neurotoxin polypeptide of a).

14. The kit of any one of claims 11 to 13, wherein the components of the kit are comprised by separate vials.

15. The kit of claim 11 or 12, wherein calculating of the amount of processed Clostridial Neurotoxin polypeptide comprises subtracting the determined amount of the second detection complex from the determined amount of the first detection complex.

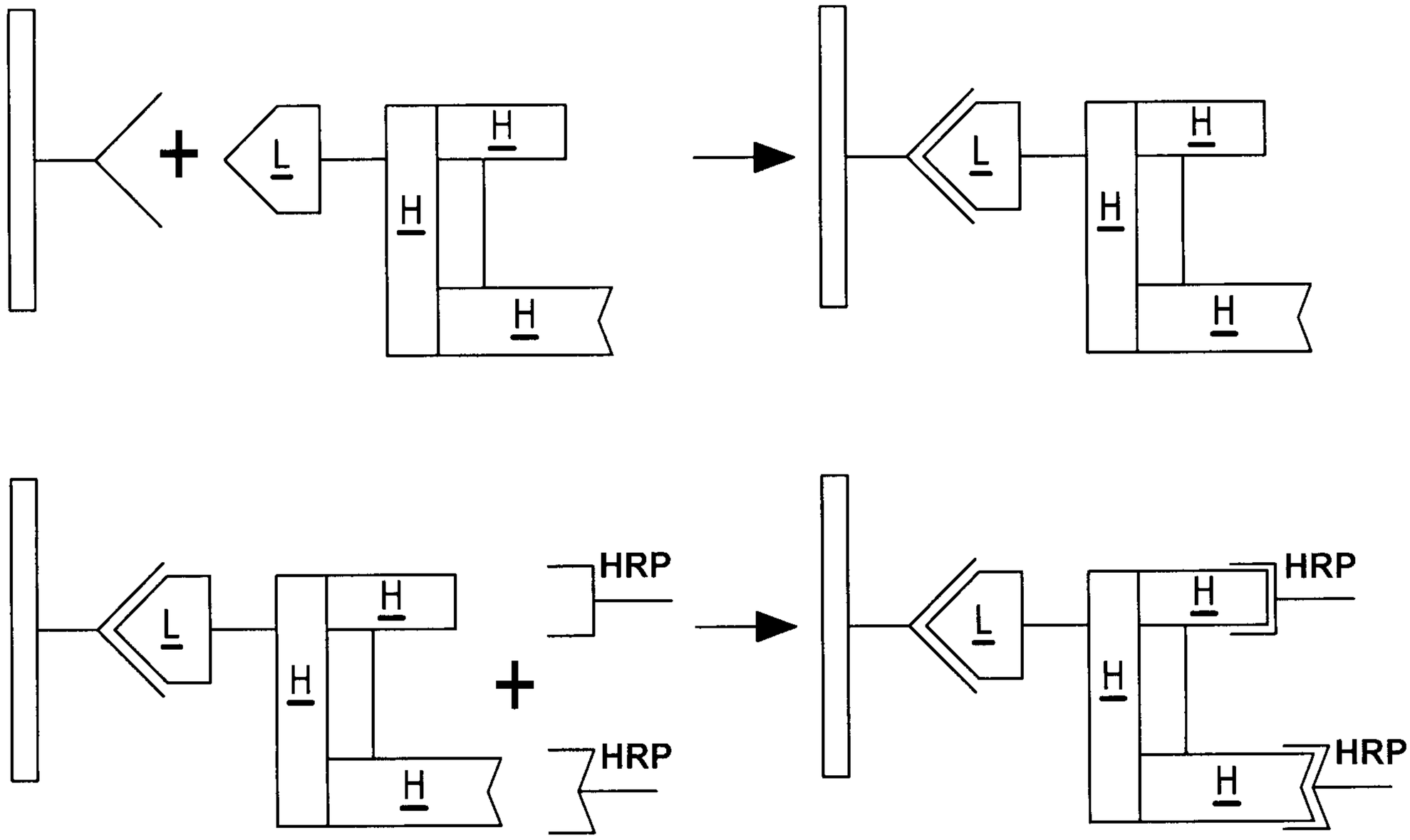


Fig. 1

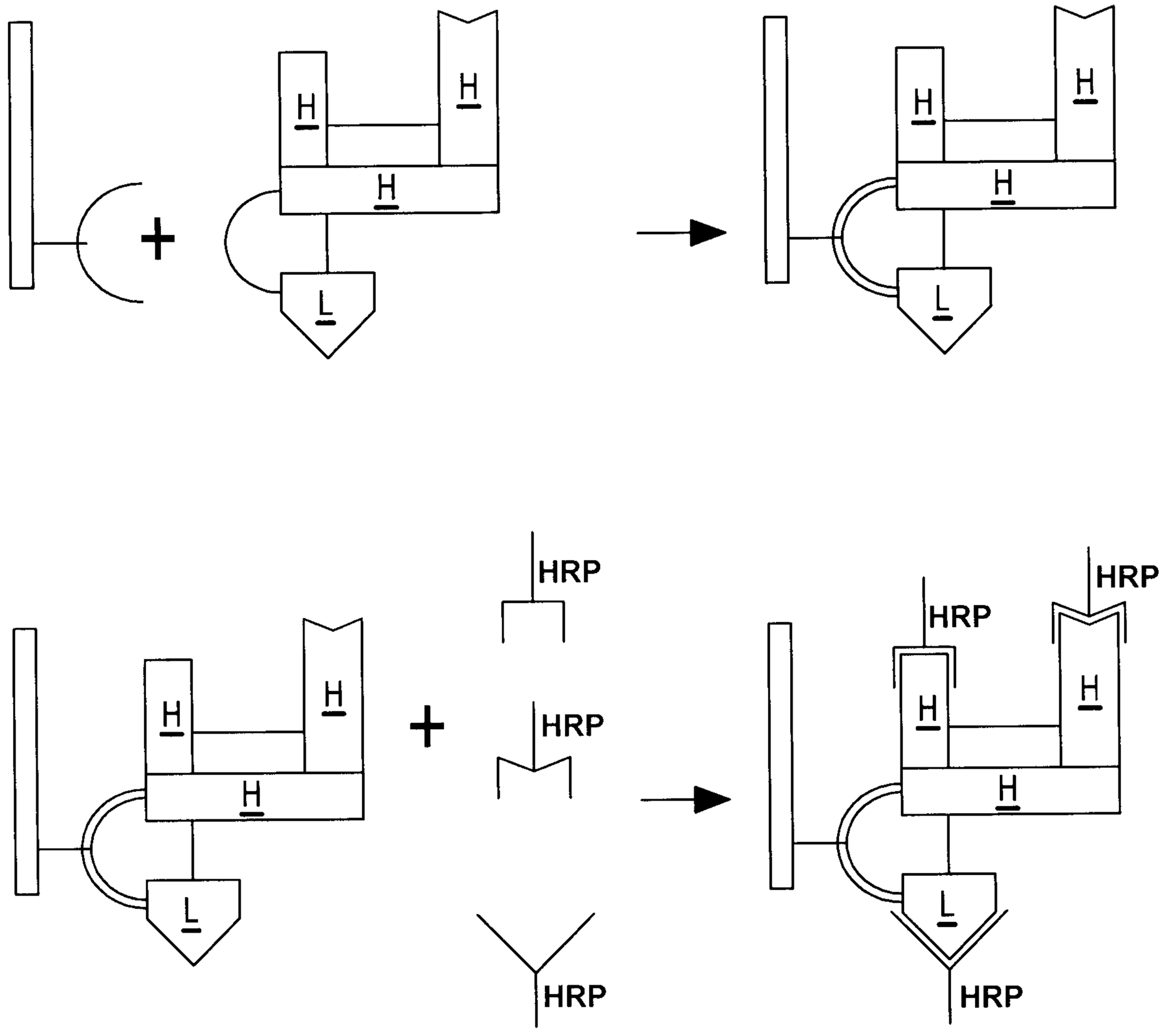


Fig. 2

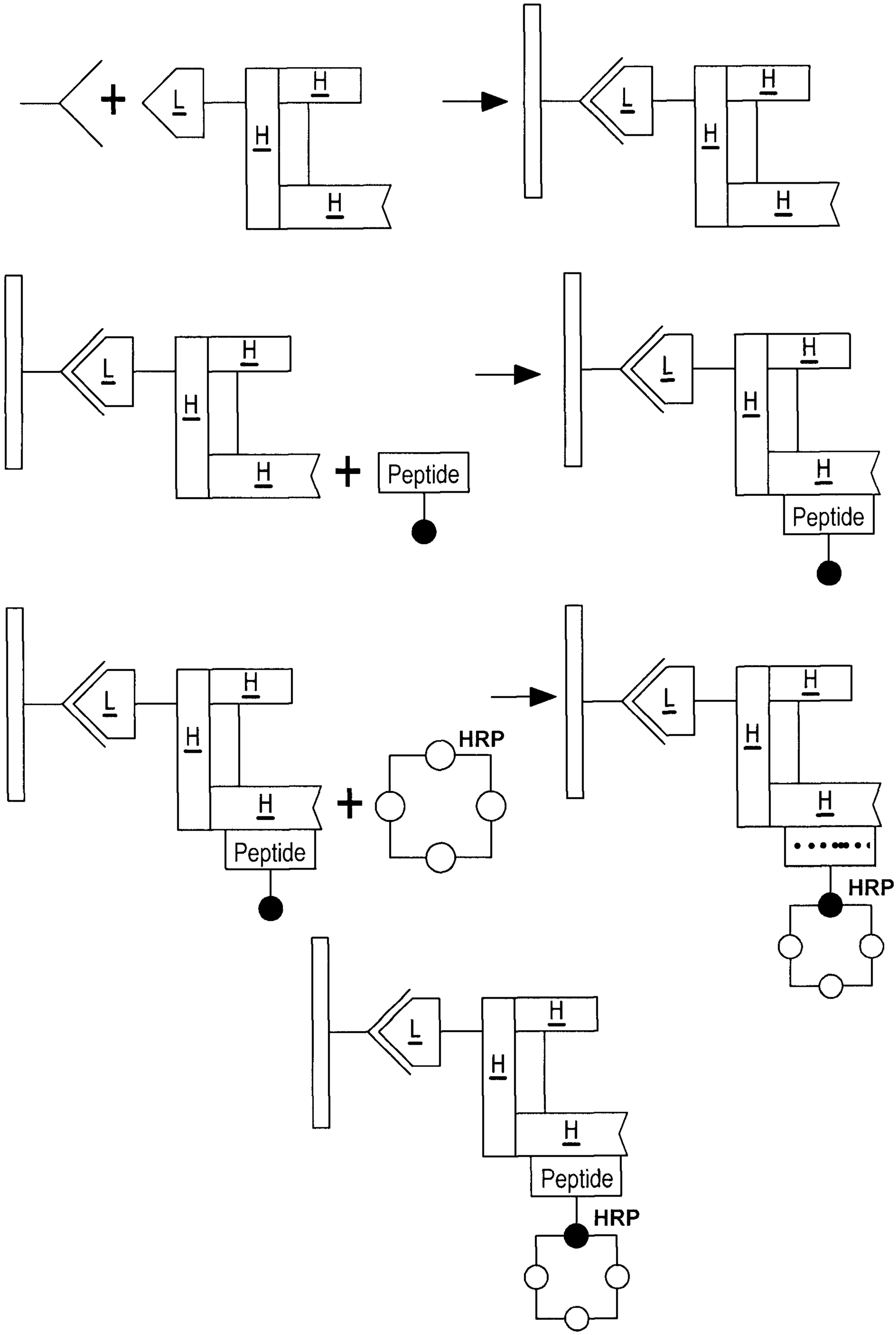


Fig. 3

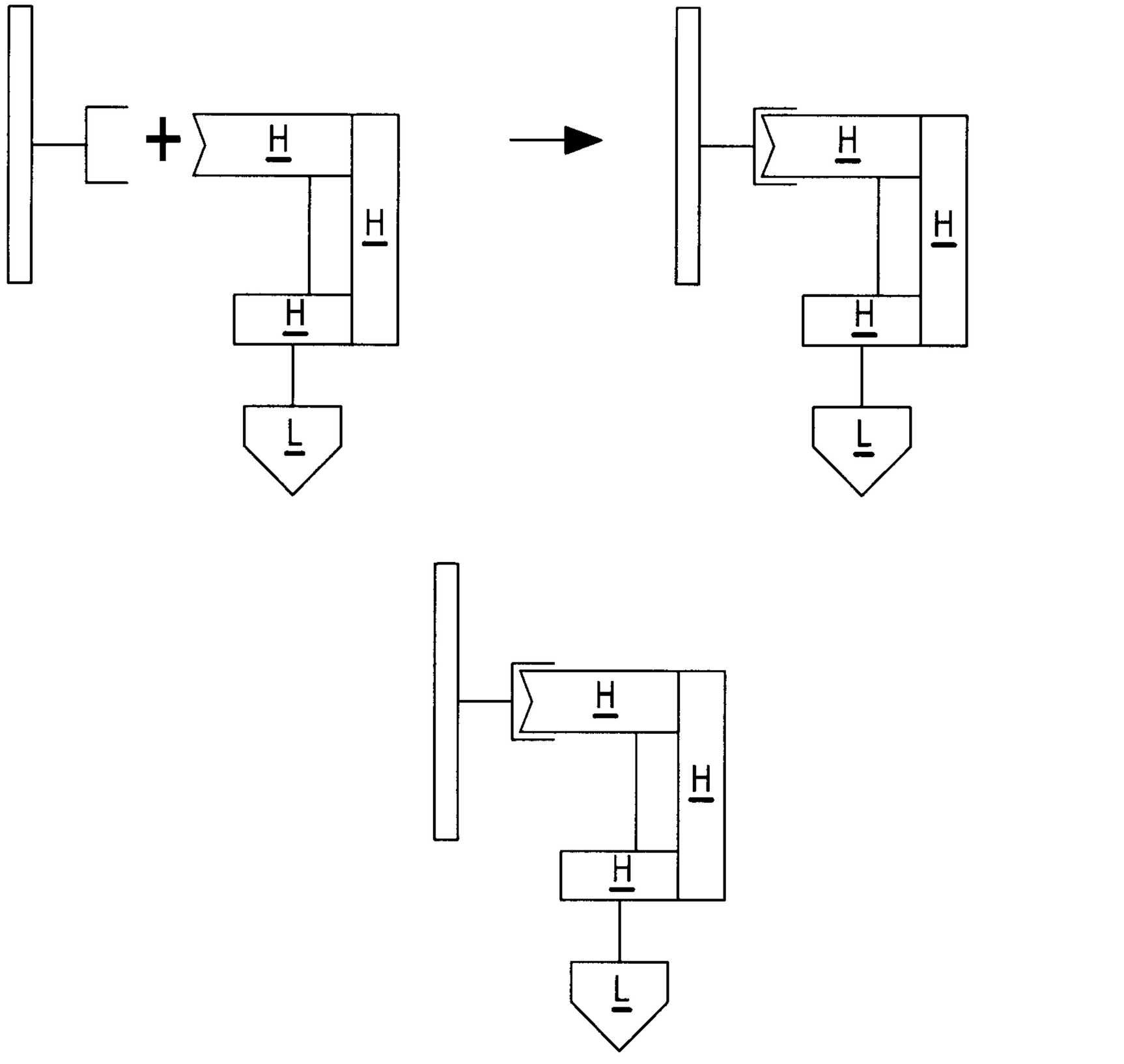
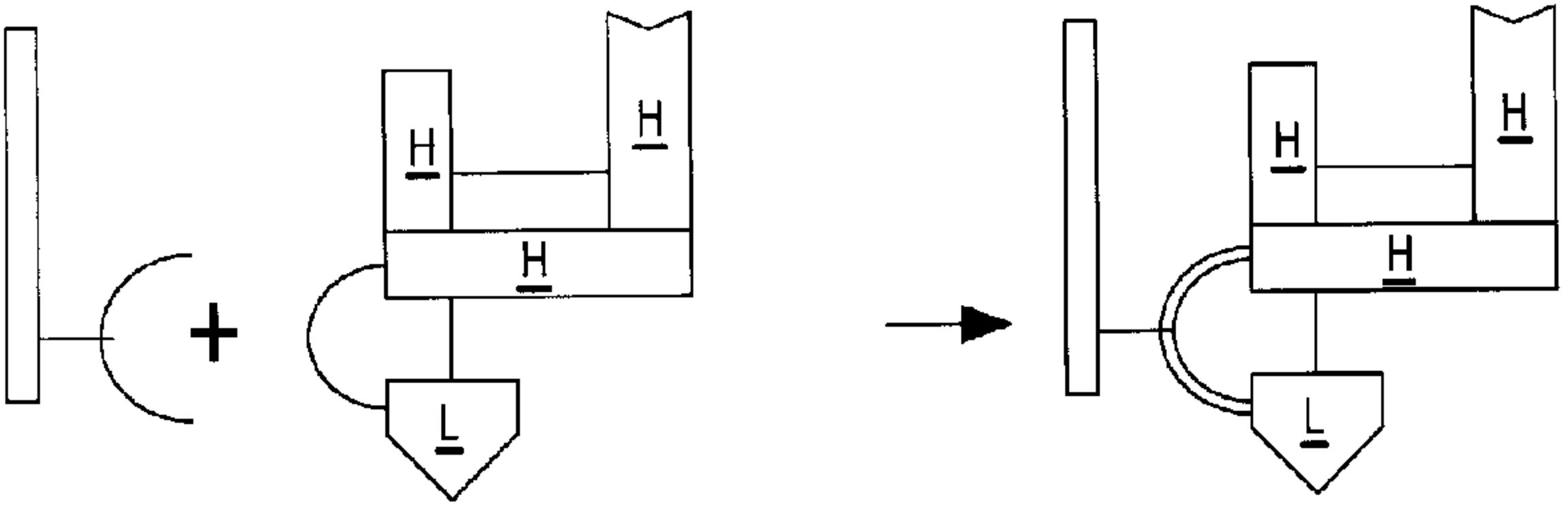
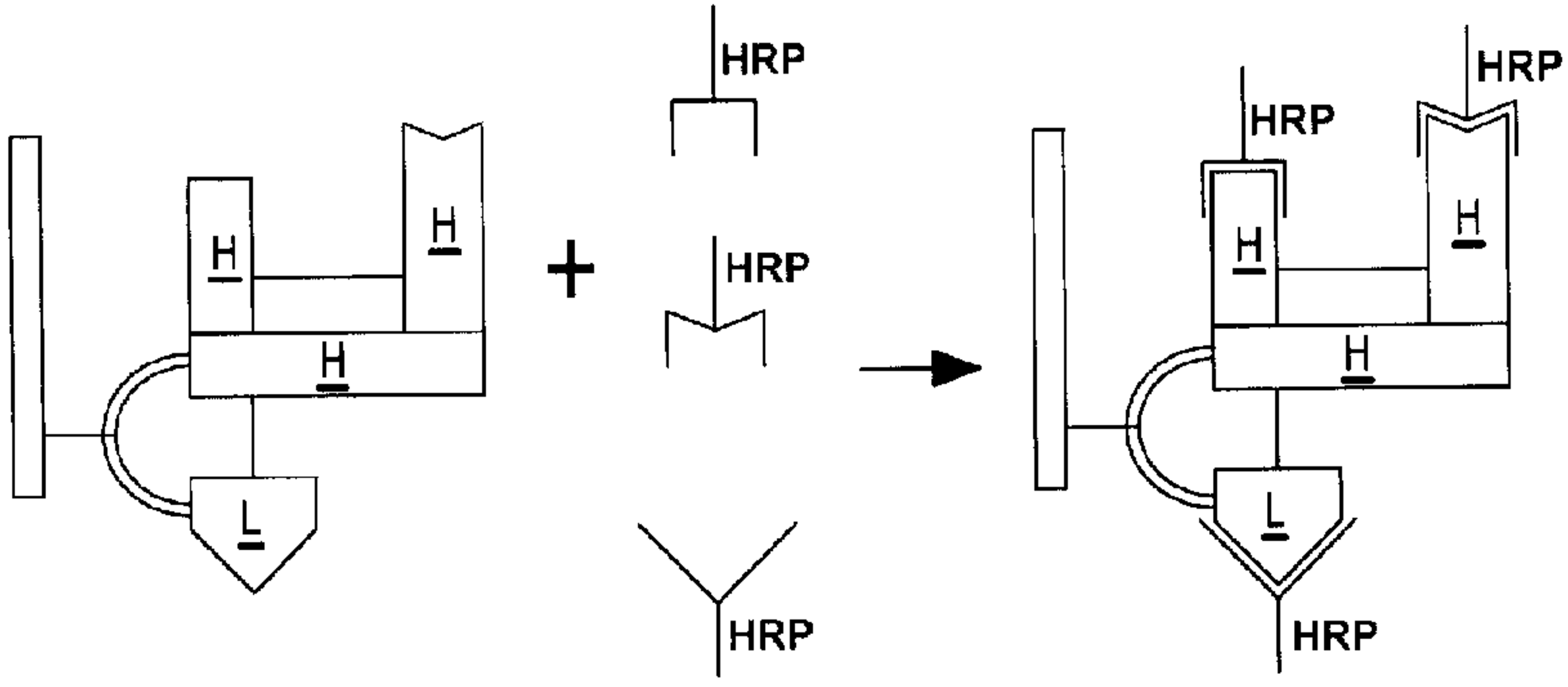

H
H
H
L
H
H
H
L
H
H
H
L
Peptide-Arg-
p-Nitrophenylaniline
Peptide-Arg +
p-Nitrophenylaniline (yellow)

Fig. 4


H
H
H
L
H
H
H


H
H
H
L
HRP
HRP
HRP
HRP
H
H
H
L
HRP
HRP