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(54) **Title**: Polypeptide mixes with antibacterial activity

(57) **Abstract**: The invention relates to the field of microbiology, specifically to a combination of a source of a first enzymatic active domain and a source of a second enzymatic active domain and to a composition comprising said combination. The invention further relates to a composition comprising said combination for use as a medicament, to the use of said composition as an antimicrobial agent and to a method for controlling microbial contamination in a food-or feed product, on and/or in food-or feed processing equipment, on and/or in food-or feed containers.

# POLYPEPTIDE MIXES WITH ANTIBACTERIAL ACTIVITY

## Field of the invention

The invention relates to the field of microbiology, specifically to a combination of a source of a first enzymatic active domain and a source of a second enzymatic active domain, a polypeptide, a polynucleotide and to a composition comprising said combination, polypeptide and/or polynucleotide. The invention further relates to a composition comprising said combination, polypeptide and/or polynucleotide for use as a medicament, to the use of said composition, polypeptide and/or polynucleotide as an antimicrobial agent and to a method for controlling microbial contamination in a food- or feed product, on and/or in food- or feed processing equipment, on and/or in food- or feed containers.

## Background of the invention

*Staphylococcus aureus* is a major human pathogen frequently causing serious infectious diseases and food poisoning. Its treatment becomes more and more difficult because of emerging antibiotic resistant strains. Endolysins from phages infecting *Staphylococcus aureus* have been shown to potentially control these pathogens to a certain extent and can be used for their specific detection. In most cases, major obstacles in the application of endolysins targeting *Staphylococcus* species are low enzyme activity, difficult production in large quantities and/or protein stability.

Accordingly, there is still a need for new antimicrobials with improved characteristics on for example antimicrobial activity and/or stability.

## Description of the invention

In a first aspect, the present invention provides a combination of a source of a first enzymatic active domain and a source of a second enzymatic active domain, wherein said first and second enzymatic active domains exhibit distinct target bond specificities and are comprised on a distinct first and second polypeptide, *i.e.* said first enzymatic active domain is comprised on a first polypeptide and said second enzymatic domain is comprised on a second polypeptide, wherein said first and second polypeptide each have a distinct amino acid sequence. In addition, the present invention provides a combination of a source of a first enzymatic active domain, a source of a

second enzymatic active domain and a source of a third enzymatic active domain, wherein said first, second and third enzymatic active domain exhibit distinct target bond specificities and are comprised on a distinct first, second and third polypeptide, *i.e.* said first enzymatic active domain is comprised on a first polypeptide, said second enzymatic domain is comprised on a second polypeptide, and said third enzymatic domain is comprised on a third polypeptide, wherein said first, second and third polypeptide each have a distinct amino acid sequence. Furthermore, the present invention provides a combination of a source of a first enzymatic active domain, a source of a second enzymatic active domain, a source of a third enzymatic active domain, and a source of a further enzymatic active domain, wherein said first, second, third and further enzymatic active domain exhibit distinct target bond specificities and are comprised on a distinct first, second, third and further polypeptide, *i.e.* said first enzymatic active domain is comprised on a first polypeptide, said second enzymatic domain is comprised on a second polypeptide, said third enzymatic domain is comprised on a third polypeptide, and said further enzymatic active domain is comprised on a further polypeptide, wherein said first, second, third and further polypeptide each have a distinct amino acid sequence. A further enzymatic active domain is meant herein as a fourth, fifth, sixth, seventh, eighth, ninth, tenth or more enzymatic active domain, preferably a fourth enzymatic active domain. A further polypeptide is meant herein as a fourth, fifth, sixth, seventh, eighth, ninth, tenth or more polypeptide, preferably a fourth polypeptide.

Most native *Staphylococcus* bacteriophage endolysins exhibiting peptidoglycan hydrolase activity consist of a C-terminal cell wall-binding domain (CBD), a central N-acetylmuramoyl-L-Alanine amidase domain, and an N-terminal Alanyl-glycyl endopeptidase domain with cysteine, histidine-dependent amidohydrolases/peptidase (CHAP) homology, or in case of Ply2638, of an N-terminal glycyl-glycine endopeptidase domain with Peptidase_M23 homology, the latter three domains exhibiting peptidoglycan hydrolase activity each with distinct target bond specificity and generally named herein as enzymatically active domains. Within the present invention, a first enzymatic domain according to the present invention has catalytic activity to hydrolyse a specific target bond, which is different from the target bond hydrolysed by a second and optionally a third and/or further enzymatic active domain according to the present invention. Furthermore, within the present invention, a second

enzymatic domain according to the present invention has catalytic activity to hydrolyse a specific target bond, which is different from the target bond hydrolysed by a first and optionally a third and/or further enzymatic active domain according to the present invention.

The inventors surprisingly found that simultaneous application of two or more enzymatically active domains with distinct target bond specificities confer synergistic effects. Surprisingly, this works not only when enzymatically active domains with different specificities are located on the same molecule as in native *Staphylococcus* endolysins, but works also when the enzymatically active domains with different specificities are separated on distinct polypeptides.

The benefit of having distinct enzymatic active domains located on separate individual polypeptides is that the resulting polypeptides are smaller which can be more easily produced. Furthermore, these smaller polypeptides have better diffusion properties in specific environments and can be more resistant to degradation and feature higher thermostability. Another advantage is that independent distinct enzymatic active domains located on separate distinct polypeptide molecules can be mixed and pooled in variable compositions, at a ratio that is best suited to hydrolyse the specific bacterial target cells. The combination according to the invention can be supplemented and/or complemented by the use of virtually any functional enzymatic active domain with virtually any target bond specificity from many different origins including phage lysins, bacteriocins, autolysins, or any other cell wall lytic enzymes.

Within the context of the present invention 'a combination' means that a source of a first enzymatic active domain and a source of a second enzymatic active domain are contemplated and encompassed. In addition, within the context of the present invention 'a combination' means that a source of a first enzymatic active domain, a source of a second enzymatic active domain and optionally a source of a third and/or further enzymatic active domain are contemplated and encompassed. Each source may be together or present together or combined together or physically in contact with the other source forming one single composition. Each source may alternatively be comprised within a distinct composition. However the invention provides the insight that both sources of a first and a second enzymatic active domain are needed or are used in order to get an effect of the present invention as defined herein. If each source is not present in a same single composition, each source and/or each distinct

composition comprising a source of a combination according to the present invention may be used sequentially or simultaneously.

'A source of a first enzymatic active domain', 'a source of a second enzymatic active domain', 'a source of a third enzymatic active domain' and 'a source of a further enzymatic active domain' preferably comprises a protein-based source, *i.e.* a polypeptide, a protein, digest of a protein and/or fragment of a protein or digest, or a source not being protein based, *i.e.* a nucleic acid encoding a protein or derived peptide or protein fragment. Below we defined preferred sources of a first enzymatic active domain, a source of a second enzymatic active domain, a source of a third enzymatic active domain and a source of a further enzymatic active domain that are encompassed by the invention. Since the invention relates to a combination of a source of a first enzymatic active domain, a source of a second enzymatic active domain and optionally a source of a third and/or further enzymatic active domain, each of the sources of a first enzymatic active domain defined herein may be combined with each of the sources of a second and optionally third and/or further enzymatic active domain defined herein. It is also encompassed by the present invention to use a combination of a source of a first enzymatic active domain being protein-based with a source of a second and optionally a third and/or further enzymatic active domain being not protein-based, and vice versa.

'An enzymatic active domain' is defined herein is a domain having lytic activity, preferably exhibiting peptidoglycan hydrolase activity. Lytic activity of a first, second, third and/or further enzymatic active domain according to the present invention comprised on a distinct first, second, third and/or further polypeptide according to the present invention can be assessed by methods well known by the person skilled in the art. In an embodiment, lytic activity is assessed spectrophotometrically by measuring the drop in turbidity of substrate cell suspensions. Turbidity is assessed by measuring optical density at a wavelength of 595 nm, typically a culture as turbid when it exhibits an optical density of at least 0.3 OD at a wavelength of 595 nm. Preferably, lytic activity is assessed spectrophotometrically measuring the drop in turbidity of a *S. aureus* suspension, wherein turbidity is quantified by measuring $OD_{595}$ spectrophotometrically (Libra S22, Biochrom). More preferably, 200 nM of a first, second and/or third polypeptide as identified herein is incubated together with an *S. aureus* suspension having an initial $OD_{595}$ of 1 ± 0.05, as assessed spectrophotometrically (Libra S22, Biochrom), in PBS buffer pH 7.4, 120 mM sodium

chloride for 30 min at 37°C. The drop in turbidity is calculated by subtracting the $OD_{595}$ after 30 min of incubation from the $OD_{595}$ before 30 min of incubation. Within the context of the invention a first, second and/or third polypeptide will be said to have lytic activity if, when using this assay, a drop in turbidity of at least 10, 20, 30, 40, 50 or 60% is detected. Preferably, a drop in turbidity of at least 70% is detected. Preferably, the invention relates to a first, second, third and/or further polypeptide which exhibits a lytic activity of at least 30, 40, 50, 60, 70, 80, 90, 100, 150 or 200% or more of a lytic activity of *S. aureus* bacteriophage Φ2638a endolysin (Ply2638 endolysin identified by SEQ ID NO: 2) encoded by SEQ ID NO: 1.

'Exhibit distinct target bond specificities' is meant herein as exhibiting enzymatic activity against a target bond by any of a first, second, third or further enzymatic active domain according to the present invention which is distinct from the target bond to which any of the other of said first, second, third or further enzymatic active domain exhibits enzymatic activity.

'Comprised on distinct polypeptides' is meant herein as any of said first, second and optionally third and/or further enzymatic active domain is comprised on a polypeptide which is distinct from the polypeptide that any of the other of said first, second and optionally third and/or further enzymatic active domain is comprised on.

A polypeptide according to the present invention preferably is an isolated polypeptide. A nucleic acid according to the present invention preferably is an isolated nucleic acid. A nucleic acid construct according to the present invention preferably is an isolated nucleic acid construct.

In a preferred embodiment, a polypeptide according to present invention comprises a sequence encoding a tag for ease of purification. Preferably, said tag is selected from, but is not limited to, the group consisting of a FLAG-tag, poly(His)-tag, HA-tag and Myc-tag. More preferably said tag is a 6xHis-tag. Even more preferably, said tag is an N-terminal 6xHis-tag (indicated herein as HXa) identical to SEQ ID NO: 74 and encoded by SEQ ID NO: 73).

Preferably, a distinct target bond according to the present invention is an essential bond in a peptidoglycan layer of a bacterial cell, preferably said bacterial cell is a *Staphylococcus*. An essential bond in a peptidoglycan layer of a gram-positive bacterial cell is defined herein as a linkage within said peptidoglycan that is essential for said peptidoglycan to provide said bacterial cell shape and a rigid structure

resistance to osmotic shock. Preferably, said essential bond in a peptidoglycan layer of a gram-positive bacterial cell is a bond between a D-alanine of the stem peptide and a glycine of the cross-bridge peptide (defined herein also as a bond between an N-terminal alanine and a glycine), a bond in a pentaglycin cross-bridge (defined herein also as a pentaglycin bridge glycyl-glycyl bond, a bond between an N-acetylmuramoyl and an L-alanine or a bond between an N-acetylmuramine and a N-acetylglucosamine or between a N-acetylglucosamine and an N-acetylmuramine (Figure 1). Other preferred essential bonds in a peptidoglycan layer of a gram-positive bacterial cell are a bond in a gamma-glutamyl stem peptide, a bond between a L-Alanyl-iso-D-glutamic acid in a stem peptide and a bond between an iso-D-glutamic acid-L-Lysine in a stem peptide.

Preferably, a first, a second and optionally a third and/or further enzymatic active domain according to the present invention is a domain selected from the group consisting of a cysteine, histidine-dependent amidohydrolases/peptidase (CHAP) domain, an endopeptidase domain, and an amidase domain. Moreover, preferably, said first, second, third and/or further enzymatic active domain is a domain selected from the group consisting of a cysteine, histidine-dependent amidohydrolases/peptidase (CHAP) domain, an endopeptidase domain, an amidase domain, and a glycosylhydrolase domain. Said glycosylhydrolase domain can be a muramidase domain or a glycosaminidase domain.

Preferably, said CHAP domain cleaves a bond between an N-terminal Alanyl and a glycyl within a peptidoglycan layer. More preferably, said CHAP domain specifically cleaves a bond between an N-terminal Alanyl and a glycyl within a peptidoglycan layer. Preferably, said endopeptidase domain cleaves pentaglycin bridge glycyl-glycyl bond within a peptidoglycan layer. More preferably, said endopeptidase domain specifically cleaves pentaglycin bridge glycyl-glycyl bond within a peptidoglycan layer. Preferably, said amidase domain cleaves a bond between a central N-acetylmuramoyl and an L-Alanine within a peptidoglycan layer. More preferably, said amidase domain specifically cleaves a bond between a central N-acetylmuramoyl and an L-Alanine within a peptidoglycan layer. Preferably, said muramidase domain cleaves a bond between an N-acetylmuramine and a N-acetylglucosamine within a peptidoglycan layer. More preferably, said muramidase domain specifically cleaves a bond between an N-acetylmuramine and a N-acetylglucosamine within a peptidoglycan

layer. Preferably, said glucosaminidase domain cleaves a bond between a N-acetylglucosamine and an N-acetylmuramine within a peptidoglycan layer. More preferably, said glucosaminidase domain specifically cleaves a bond between a a N-acetylglucosamine and an N-acetylmuramine within a peptidoglycan layer. Preferably said peptidoglycan layer is of a gram positive bacterial cell, preferably of a *Staphylococcus*, most preferably of a *Staphylococcus aureus*. Preferably, the cleavage of a bond by an enzymatic active domain as defined herein is specific if such a bond is hydrolysed at least 2, 10, 50 or a 100 times more efficient with said enzymatic active domain as compared to the hydrolyses of any other bond as defined herein above with said enzymatic active domain.

Preferably, a CHAP domain encompassed within the present invention originates from *Staphylococcus* phage K and/or *Staphylococcus* phage Twort. Preferably, a CHAP domain encompassed within the present invention, is a domain that has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 10 or 12. Preferably, an endopeptidase domain encompassed within the present invention originates from *S. aureus* bacteriophage Φ2638a and/or *S. simulans*. Preferably, an endopeptidase domain encompassed by the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 14 or 16. Preferably, an amidase domain encompassed within the present invention originates from *S. aureus* bacteriophage Φ2638a. Preferably an amidase domain of the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 18.

Preferably, a first, second, third and/or further polypeptide according to the present invention comprises a different multiplicity of a first, second, third and/or further enzymatic active domain according to the present invention. A “multiplicity” is herein defined as a number of copies. A “different multiplicity” is defined herein as a multiplicity or number of copies of a specific enzymatic active domain of the invention, *i.e.* a first, second, third or further enzymatic active domain as identified herein, comprised within a specific polypeptide of the invention, *i.e.* a first, second, third or further polypeptide as identified herein, to be different form a multiplicity or number of copies of that same enzymatic active domain within another polypeptide of the combination of the invention. For example, a combination of the present invention

comprises a first polypeptide comprising a specific number of copies of a first enzymatic active domain, and a second polypeptide comprising a different number of copies of said first enzymatic active domain. Furthermore, said first polypeptide of said exemplified combination of the present invention may further comprise a specific number of copies of second enzymatic active domain, which is different from the number of copies of said second enzymatic active domain as comprised on said second polypeptide of said combination. Furthermore, any further polypeptide of said exemplified combination of the present invention may comprise a number of copies of further enzymatic active domain, which is different from the number of copies of said further enzymatic active domain as comprised on said first and second polypeptide of said combination. Although a combination of distinct polypeptides each comprising a single distinct enzymatic active domain showed synergistic lytic activity as compared to the lytic activity of each separate polypeptide, it was surprisingly found by the present inventors that polypeptides comprising a multiplicity of enzymatic active domains show superior lytic activity as compared to polypeptides comprising a single enzymatic active domain.

Moreover, a combination of distinct enzymatic domains on distinct polypeptides wherein at least one of said distinct polypeptides comprises a multiplicity of enzymatic active domains was found superior over a combination wherein all said distinct polypeptides comprise a single distinct enzymatic active domain. Moreover, a combination according to the present invention, wherein a first, second, third and/or further polypeptide according to the present invention comprise a multiplicity of a first, second, third and/or further enzymatic active domain according to the present invention, respectively, was found superior over a combination according to the present invention, wherein said first, second, third and/or further polypeptide comprise a single copy of said first, second, third and/or further enzymatic active domain, respectively, and preferably wherein said multiplicity, as defined herein, is 2, *i.e.* a duplicate. In a preferred embodiment, the synergistic effect of a combination according to the present invention, wherein a first, second, third and/or further polypeptide according to the present invention comprise a multiplicity of a first, second, third and/or further enzymatic active domain according to the present invention, respectively, was found superior over a combination according to the present invention, wherein said first, second, third and further polypeptide comprise a single copy of said first, second, third

and further enzymatic active domain, respectively, and preferably wherein said multiplicity, as defined herein below, is 2, *i.e.* a duplicate.

Preferably, a first and/or second polypeptide according to the present invention comprises a different multiplicity of a first and/or second enzymatic active domain according to the present invention. Multiplicity of said first and second domain is defined as previously herein as a number of copies, preferably indicated by *k*, *l*, *n* and *p,* of said first and second domain indicated as follows:

*k* indicates the number of copies of said first enzymatic active domain on said first polypeptide;

*l* indicates the number of copies of said second enzymatic active domain on said first polypeptide;

*n* indicates the number of copies of said first enzymatic active domain on said second polypeptide;

*p* indicates the number of copies of said second enzymatic active domain on said second polypeptide;

and wherein *k* and *p* are independent integers from 1-10, 1-9, 1-8, 1-7, 1-6, 1-5, 1-4, 1-3, or preferably 1-2, and *l* and *n* are independent integers from 0-10, 0-9, 0-8, 0-7, 0-6, 0-5, 0-4, 0-3, or preferably 0-2, and wherein *k* is a different integer than *n* and/or *l* is a different integer than *p*, most preferably *k* and *p* are 2 and *l* and *n* are 0.

Preferably, a first, second and third polypeptide of the present invention comprise a different multiplicity of a first, second and third enzymatic active domain according to the present invention.

Multiplicity of said first, second and third domain is defined as previously herein as a number of copies, preferably indicated by *k*, *l*, *m*, *n*, *p*, *q*, *r*, *s* and *t,* of said first, second and third domain indicated as follows:

*k* indicates the number of copies of said first enzymatic active domain on said first polypeptide;

*l* indicates the number of copies of said second enzymatic active domain on said first polypeptide;

*m* indicates the number of copies of said third enzymatic active domain on said first polypeptide;

*n* indicates the number of copies of said first enzymatic active domain on said second polypeptide;

*p* indicates the number of copies of said second enzymatic active domain on said second polypeptide;

*q* indicates the number of copies of said third enzymatic active domain on said second polypeptide;

*r* indicates the number of copies of said first enzymatic active domain on said third polypeptide;

*s* indicates the number of copies of said second enzymatic active domain on said third polypeptide;

*t* indicates the number of copies of said third enzymatic active domain on said third polypeptide;

and wherein *k*, *p* and *t* are independent integers from 1-10, 1-9, 1-8, 1-7, 1-6, 1-5, 1-4, 1-3, or preferably 1-2, and *l*, *m*, *n*, *q*, *r*, and *s* are independent integers from 0-10, 0-9, 0-8, 0-7, 0-6, 0-5, 0-4, 0-3, or preferably 0-2, and wherein *k* is a different integer than *n* and/or *r*, and/or *l* is a different integer than *p* and/or *s*, and/or *t* is a different integer than *m* or *q*, most preferably *k*, *p* and *t* are 2 and *l*, *m*, *n*, *q*, *r*, and *s* are 0 .

Preferably, a first, second, third and further polypeptide of the present invention comprise a different multiplicity of a first, second, third and further enzymatic active domain according to the present invention. Multiplicity of said further enzymatic active domain in view of said first, second and third enzymatic active domain is to be construed herein in an analogous manner as defined herein above for a first, second and third enzymatic active domain.

Preferably a first, second, third or further polypeptide according to the present invention has a length of at least 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320 or 330 amino acids and/or a length of at most 850, 800, 750, 700, 650, 600, 550, 500, 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380 or 370 amino acids. More preferably, a first, second or third polypeptide according to the present invention has a length of 140-850, 140-800, 140-750, 140-700, 140-650, 140-600, 140-550 140-500, 140-490, 140-480, 140-470, 140-460, 140-450, 140-440, 140-430, 140-420, 140-410, 140-400, 140-390, 140-380, 140-370, 150-850, 160-850, 170-850, 180-850, 190-850, 200-850, 210-850, 220-850, 230-850, 240-850,

250-850, 260-850, 270-850, 280-850, 290-850, 300-850, 310-850, 320-850 or 330-850 amino acids.

Preferably a first and second polypeptide according to the present invention each have a length of at least 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320 or 330 amino acids and/or a length of at most 800, 850, 700, 650, 600, 550, 500, 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380 or 370 amino acids. More preferably, a first and second polypeptide according to the present invention each have a length of 140-850, 140-800, 140-750, 140-700, 140-650, 140-600, 140-550 140-500, 140-490, 140-480, 140-470, 140-460, 140-450, 140-440, 140-430, 140-420, 140-410, 140-400, 140-390, 140-380, 140-370, 150-850, 160-850, 170-850, 180-850, 190-850, 200-850, 210-850, 220-850, 230-850, 240-850, 250-850, 260-850, 270-850, 280-850, 290-850, 300-850, 310-850, 320-850 or 330-850 amino acids.

Preferably a first, second and third polypeptide according to the present invention each have a length of at least 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320 or 330 amino acids and/or a length of at most 800, 850, 700, 650, 600, 550, 500, 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380 or 370 amino acids. More preferably, a first, second and third polypeptides according to the present invention each have a length of 140-850, 140-800, 140-750, 140-700, 140-650, 140-600, 140-550 140-500, 140-490, 140-480, 140-470, 140-460, 140-450, 140-440, 140-430, 140-420, 140-410, 140-400, 140-390, 140-380, 140-370, 150-850, 160-850, 170-850, 180-850, 190-850, 200-850, 210-850, 220-850, 230-850, 240-850, 250-850, 260-850, 270-850, 280-850, 290-850, 300-850, 310-850, 320-850 or 330-850 amino acids.

Preferably a first, second, third and further polypeptide according to the present invention each have a length of at least 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320 or 330 amino acids and/or a length of at most 800, 850, 700, 650, 600, 550, 500, 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380 or 370 amino acids. More preferably, a first, second, third and further polypeptides according to the present invention each have a length of 140-850, 140-800, 140-750, 140-700, 140-650, 140-600, 140-550 140-500, 140-490, 140-480, 140-470, 140-460, 140-450, 140-440, 140-430, 140-420, 140-410, 140-400, 140-390, 140-380, 140-370, 150-850, 160-850, 170-850, 180-850, 190-850, 200-850, 210-850, 220-

850, 230-850, 240-850, 250-850, 260-850, 270-850, 280-850, 290-850, 300-850, 310-850, 320-850 or 330-850 amino acids.

An embodiment provides a combination of a source of a first and a second enzymatic active domain according to the present invention, wherein said first and second enzymatic active domains are comprised on distinct, first and second polypeptides of the present invention, wherein said first polypeptide is free of said second enzymatic active domain and said second polypeptide is free of said first enzymatic active domain. Moreover, provided is a combination according to the present invention, wherein *l and n* are 0.

Another embodiment provides a combination of a source of a first, second and third enzymatic active domain according to the present invention, wherein said first, second and third enzymatic active domains are comprised on distinct, first, second and third polypeptides, wherein said first polypeptide is free of said second and third enzymatic active domain, said second polypeptide is free of said first and third enzymatic active domain, and said third polypeptide is free of said first and second enzymatic active domain. Moreover, provided is a combination according to the present invention, wherein *l*, *m*, *n*, *q*, *r* and *s* are 0. Even more preferably, the present invention provides a combination according to the present invention, wherein *l*, *m*, *n*, *q*, *r* and *s* are 0 and *k*, *p* and *t* are 2.

Another embodiment provides a combination of a source of a first, second, third and further enzymatic active domain according to the present invention, wherein said first, second, third and further enzymatic active domains are comprised on a distinct, first, second, third and further polypeptide, respectively, wherein

preferably said first polypeptide is free of said second, third and further enzymatic active domain;

preferably said second polypeptide is free of said first, third and further enzymatic active domain;

preferably said third polypeptide is free of said first, second and further enzymatic active domain; and,

preferably said further polypeptide is free of said first, second and third enzymatic active domain.

Preferably said first, second, third and further enzymatic active domain are comprised within said first, second, third and further polypeptide, respectively, in

duplicate, *i.e.* wherein the multiplicity as identified herein is 2.Also encompassed is a combination according to the present invention, wherein a first, second and/or third polypeptide according to the present invention are not free of a first, second and/or third enzymatic active domain according to the present invention, but said first, second and/or third polypeptide differ in multiplicity of said first, second and/or third enzymatic active domain. Moreover, encompassed is a combination according to the present invention, wherein at least one of *k*, *l*, *m*, *n p*, *q*, *r*, s or *t* is 2 and wherein any of the other *k*, *l*, *m*, *n p*, *q*, *r*, s and/or *t* is 1 or 0.

Preferred is a combination according to the present invention, wherein a polypeptide according to the present invention further comprises a cell wall-binding domain. Moreover, a first, second, third and/or further polypeptide according to the present invention each comprising at least one enzymatic active domain as defined herein, further comprise a cell wall-binding domain. A cell wall-binding domain of the present invention is defined as an element, preferably a polypeptide within said distinct polypeptide, that directs said distinct polypeptide to the bacterial wall of the cell. Preferably, a cell wall-binding domain of the present invention is an element, preferably a polypeptide within said distinct polypeptide, that directs said distinct polypeptide to the peptidoglycan cell wall of a gram-positive bacterial cell, preferably the peptidoglycan cell wall of a *Staphylococcus* bacterial cell.

Binding of a domain to the peptidoglycan cell wall of *Staphylococcus* genera may be assessed using assays well known to the person skilled in the art. In a preferred embodiment, an immunohistochemical technique and/or a gene fusion technique resulting in labelled constructs are used for assessing specific binding of peptides, polypeptides or proteins to the peptidoglycan cell wall of *Staphylococcus* genera. Quantification methods of signals used in the above mentioned immunohistochemical or fusion techniques are well known in the art.

In one embodiment, *Staphylococcus* peptidoglycan cell wall-binding is quantified using a fluorescent fusion construct comprising a cell wall-domain of interest. Such a cell wall-binding assay is described in detail by Loessner et al (Molecular Microbiology 2002, 44(2): 335–349). In this assay a solution comprising said fluorescent fusion construct or a negative control, preferably Green Fluorescent Protein (GFP), is subjected to *Staphylococcus* cells, preferably *S. aureus* cells, more preferably *S. aureus* BB255 for an indicated time period where after the cells are sedimented by

centrifugation together with the bound fluorescent fusion constructs. The fluorescent signal of the *Staphylococcus* cells exposed to a fluorescent fusion construct subtracted by the fluorescence signal of the *Staphylococcus* cells exposed to a negative control, preferably GFP, is a measure for cell binding as meant in this disclosure. Preferably, within the context of the invention, a domain is said to bind the peptidoglycan cell wall of *Staphylococcus* genera when using this assay an increase in fluorescent signal of the sedimented cells above the negative control as defined herein is detected. Preferably, the invention relates to a cell wall-binding domain which exhibits binding as defined herein of at least 50, 60, 70, 80, 90 or 100, 150 or 200% of peptidoglycan cell wall-binding of *S. aureus* bacteriophage Φ2638a endolysin (Ply2638 endolysin defined by SEQ ID NO: 2) encoded by SEQ ID NO: 1.

A cell wall-binding domain encompassed within the present invention may be any cell wall-binding domain known by the person skilled in the art. A preferred cell wall-binding domain of the present invention is a cell wall-binding domain having at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with the cell wall binding domain of *S. Simulans* lysostaphin defined herein by SEQ ID NO: 4 and encoded by SEQ ID NO: 3. Also preferred is a cell wall-binding domain isolated from a native *Staphylococcus* bacteriophage endolysin. Preferably, a cell wall-binding domain of the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with the cell wall-binding domain of *S. aureus* bacteriophage Φ2638a endolysin defined herein by SEQ ID NO: 6 and encoded by SEQ ID NO: 5. Also preferred is a cell wall-binding domain isolated from a native *Staphylococcus aureus* phage phiNM3 endolysin. Preferably, a cell wall-binding domain of the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with the cell wall-binding domain of *S. aureus* phage phiNM3 endolysin defined herein by SEQ ID NO: 8 and encoded by SEQ ID NO: 7.

Preferably, a cell wall-binding domain according to the present invention is located on the C-terminal side of the enzymatic active domain with said distinct first, second, third and/or further polypeptide. It is to be understood further that encompassed is a combination according to the present invention, wherein a distinct first, second and optionally third and/or further polypeptide according to the present invention do not only differ in their specific enzymatic active domains, but also in their specific cell

wall-binding domain. Even a combination according to the present invention, wherein a distinct first, second and optionally third polypeptide according to the present invention is free of a cell wall-binding domain according to the present invention is within the scope of the present invention. Furthermore, a combination according to the present invention, wherein one or two of a first, second and optionally third and/or further polypeptides according to the present invention are free of a cell wall binding domain is within the scope of the present invention.

Preferred is a combination according to the present invention, wherein a first, second, third and/or further polypeptide according to the present invention is a polypeptide that has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with a polypeptide selected from the group consisting of SEQ ID NO: 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70 or 72.

In a preferred embodiment, the present invention provides a combination of a source of first enzymatic active domain and a second enzymatic active domain, wherein said first and second enzymatic active domains are comprised on distinct first and second polypeptides, and wherein said first enzymatic active domain is a cysteine, histidine-dependent amidohydrolases/peptidase domain and said second enzymatic active domain is an endopeptidase domain or wherein said first enzymatic active domain is a cysteine, histidine-dependent amidohydrolases/peptidase domain and said second enzymatic active domain is amidase domain or wherein said first enzymatic active domain is an endopeptidase domain and said second enzymatic active domain is amidase domain, wherein said distinct first and second each further comprises a cell wall-binding domain, and wherein each of said distinct first and second polypeptides comprises a multiplicity of said first or second enzymatic active domain, preferably said multiplicity being 2, *i.e.* a duplicate. Moreover, in a preferred embodiment, the present invention provides a combination of a source of first and second enzymatic active domain, wherein said first and second enzymatic active domains are comprised on distinct first and second polypeptides, and wherein said first enzymatic domain is histidine-dependent amidohydrolases/peptidase domain and said second enzymatic active domain is an endopeptidase domain or said first enzymatic active domain is a cysteine, histidine-dependent amidohydrolases/peptidase domain and said second enzymatic active domain is amidase domain or said first enzymatic active domain is an

endopeptidase domain and said second enzymatic active domain is amidase domain, and wherein said first and second polypeptide each further comprise a cell wall binding domain.

In a preferred embodiment, the present invention provides a combination of a source of first enzymatic active domain and a second enzymatic active domain, wherein said first and second enzymatic active domains are comprised on distinct first and second polypeptides, and wherein said first enzymatic active domain is a cysteine, histidine-dependent amidohydrolases/peptidase domain and said second enzymatic active domain is an endopeptidase domain, and wherein said combination further comprises a source of a third enzymatic active domain comprised on a distinct third polypeptide, wherein said third enzymatic active domain is an amidase domain and said distinct first, second and third polypeptide each further comprises a cell wall-binding domain, and wherein each of said distinct first, second and third polypeptides comprises a multiplicity of said first, second or third enzymatic active domain, preferably said multiplicity being 2, *i.e.* a duplicate. Moreover, in a preferred embodiment, the present invention provides a combination of a source of first, second and third enzymatic active domain, wherein said first, second and third enzymatic active domains are comprised on distinct first, second and third polypeptides, and wherein said first enzymatic domain is histidine-dependent amidohydrolases/peptidase domain, said second enzymatic active domain is an endopeptidase domain and said third enzymatic active domain is an amidase domain, and wherein said first, second and third polypeptide each further comprise a cell wall binding domain.

Preferred is a combination according to the present invention, wherein, a first enzymatic active domain according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 10 and a second enzymatic active domain according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 16.

Preferred is a combination according to the present invention, wherein, a first enzymatic active domain according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 10 and a second enzymatic active domain according to the present invention as

at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 18.

Preferred is a combination according to the present invention, wherein, a first enzymatic active domain according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 16 and a second enzymatic active domain according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 18.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

More preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34.

More preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO:

70 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52 and a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46.

Preferred is a combination according to the present invention, wherein a first enzymatic active domain according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 10, a second enzymatic active domain according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 16 and a third enzymatic active domain according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 18.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86,

87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 32, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 44 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 26.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 26.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 36, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 48 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 30.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 32, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 26.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34,

a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 44 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 26.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 32, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 44 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 32, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 44 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84,

85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 50.

Further preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 56, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 68 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 50.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 60, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 72 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 54.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 56, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID

NO: 70 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 50.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 68 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 50.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 56, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 68 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 65, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 68 and a third polypeptide according to the present invention has at least 80, 81,

82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46 and a third polypeptide according to the present invention has at least 80, 81,

82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 52.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 58, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.

Also preferred is a combination according to the present invention, wherein a first polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 34, a second polypeptide according to the present invention as at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70 and a third polypeptide according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28.It is to be understood that a combination according to the present invention encompasses mixtures of a source of a first, a source of a second and optionally a source of a third and/or further enzymatic active domain according to in varying ratios. Preferably, a combination according to the present invention comprises a source a first and a source a second enzymatic active domain according to the present invention, wherein said first and second enzymatic active domain are present in equimolar amounts. Also preferred is a combination according to the present invention comprising a source a first, a source a second and a source a third enzymatic active domain according to the present invention, wherein said first, second and third enzymatic active domain are present in equimolar amounts. Also preferred is a combination according to the present invention comprising a source a first, a source a second, a source a third and a source a further enzymatic active domain according to the present invention, wherein said first, second, third and further enzymatic active domain are present in equimolar amounts.

In a second aspect, the present invention provides a combination according to the first aspect, wherein a source of a first, second and optionally third and/or further

enzymatic active domain according to the first aspect of the invention comprises a polypeptide. Said polypeptide may be a protein, a digest of a protein and/or a fragment of a protein or digest, which may be in a purified form or may be comprised within a crude composition, preferably of biological origin, such as a bacterial lysate, yeast lysate, fungal lysate, sonicate or fixate. Alternatively, said polypeptide may be a chemically synthesized polypeptide or a recombinant polypeptide produced *in vitro*.

Preferably, a source of said first enzymatic active domain according to the present invention comprises a first polypeptide according to the first aspect of the invention, a second enzymatic active domain according to the present invention comprises a second polypeptide according to the first aspect of the invention and optionally a third enzymatic active domain according to the present invention comprises a third polypeptide according to the first aspect of the invention and optionally a further enzymatic active domain according to the present invention comprises a further polypeptide according to the first aspect of the invention. More preferably, said source of said first enzymatic active domain consists of a first polypeptide according to the first aspect of the invention, said second enzymatic active domain consists of a second polypeptide according to the first aspect of the invention, said third enzymatic active domain consists of a third polypeptide according to the first aspect of the invention, and said further enzymatic active domain consists of a third polypeptide according to the further aspect of the invention.

An embodiment encompasses a combination according to the present invention, wherein a first, second and/or optionally third and/or further polypeptide of the present invention is a variant first, second and/or third and/or further polypeptide. A variant polypeptide may be a non-naturally occurring form of the polypeptide. A polypeptide variant may differ in some engineered way from the polypeptide isolated from its native source. A variant may be made by site-directed mutagenesis starting from the nucleotide sequence encoding a polypeptide as defined herein and indicated by SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71 or 73. Preferably, a polypeptide variant according to the present invention has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to any of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72 or 74. Preferably, in a combination according to the

present invention a polypeptide variant contains mutations that do not alter the biological function of the encoded polypeptide.

A polynucleotide according to the present invention can have at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to any of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71 or 73, or alternatively hybridise under stringent conditions with these given sequences. Stringent hybridisation conditions are those as understood in the art, e.g. hybridisation in 6 x SSC (20xSSC per 1000 ml : 175.3 g NaC1, 107.1 g sodium citrate.5H 20, pH 7.0), 0.1% SDS, 0.05% sodium pyrophosphate, 5 * Denhardt's solution and 20 µg/m1 denatured herring sperm DNA at 56°C for 18-24 hrs followed by two 30 min. washes in 5 x SSC, 0.1 % SDS at 56°C and two 30 min. washes in 2 x SSC, 0.1% SSC at 56°C.

According to a preferred embodiment, a polypeptide variant exhibits lytic or cell wall-binding activity which is the same or enhanced as compared to the lytic and/or cell wall-binding activity of SEQ ID NO: 2, as measured in an assay as earlier identified herein.

It is to be understood that a combination according to the present invention encompasses mixtures of a first, second and optionally third and/or further polypeptide according to the present invention in varying ratios. Preferably, a combination according to the present invention comprises a first, second and optionally third and/or further polypeptide according to the present invention, wherein a first and second enzymatic active domain are present in equimolar amounts. Also preferred is a combination according to the present invention comprising equimolar amounts of a first, second and optionally third and/or further polypeptide according to the present invention, wherein a first, second and third and/or further enzymatic active domain are present in equimolar amounts.

In a third aspect, the present invention provides a combination according to the first aspect of the present invention, wherein a source of a first enzymatic active domain according to the first aspect of the present invention comprises a polynucleotide encoding said first enzymatic active domain, a source of a second enzymatic active domain according to the first aspect of the present invention comprises a polynucleotide encoding said second enzymatic active domain, and

optionally a source of a third enzymatic active domain according to the first aspect of the present invention comprises a polynucleotide encoding said third enzymatic active domain and optionally a source of a further enzymatic active domain according to the first aspect of the present invention comprises a polynucleotide encoding said further enzymatic active domain. Said polynucleotide may be an RNA or DNA molecule.

Preferably, the present invention provides a combination according to the present invention, wherein a first polynucleotide encodes a first enzymatic active domain according to the present invention and a second polynucleotide encodes a second enzymatic active domain according to the present invention. Preferably, in a combination according to the present invention a first and/or second polynucleotide according to the present invention further encode a cell wall-binding domain as defined herein. Preferably, the present invention provides a combination according to the present invention, wherein a first polynucleotide encodes a first enzymatic active domain according to the present invention, a second polynucleotide encodes a second enzymatic active domain according to the present invention, a third polynucleotide encodes a third enzymatic active domain according to the present invention and a further polynucleotide encodes a further enzymatic active domain according to the present invention. Preferably, in a combination according to the present invention a first, second, third and/or further polynucleotide according to the present invention further encode a cell wall-binding domain as defined herein.

The present invention further provides a combination according to the present invention, wherein a first, second and/or optionally third and/or further polypeptide of the present invention is a chimeric first, second, third and/or further polypeptide encoded by naturally occurring or retrofitted polynucleotide constructs. A retrofitted construct is defined herein as a polynucleotide comprising heterologous nucleotide sequences. As used herein the term heterologous sequence or heterologous polynucleotide is one that is not naturally found operably linked as neighboring sequence of said first nucleotide sequence. As used herein, the term heterologous may mean recombinant. Recombinant refers to a genetic entity distinct from that generally found in nature. As applied to a nucleotide sequence or nucleic acid molecule, this means that said nucleotide sequence or nucleic acid molecule is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in the production of a construct that is distinct from a sequence or molecule

found in nature. Preferably, in a combination according to the present invention said chimeric polypeptide comprises at least a first, second or third polypeptide as defined earlier herein and further comprises at least one cell binding-domain as defined earlier herein.

An alternative embodiment provides for a combination of the present invention, wherein a chimeric polypeptide comprises an endolysin as defined herein, covalently linked to a hydrophobic pentapeptide on its C-terminus, preferably said hydrophobic pentapeptide is Phe-Phe-Val-Ala-Pro, resulting in increased bactericidal action of the endolysin especially towards gram negative bacteria as reported by Ibrahim et al., 1994 (JBC 1994 Vol . 269, p. 5053-5063).

Preferably, in a combination according to the present invention a first, second, third and/or further polynucleotide according to the present invention has a length of at least 420, 450, 480, 510, 540, 570, 600, 630, 660, 690, 720, 750, 780, 810, 840, 870, 900, 930, 960 or 990 nucleotides and/or a length of at most 2550, 2400, 2250, 2100, 1950, 1800, 1650, 1500, 1470, 1440, 1410, 1380, 1350, 1320, 1290, 1260, 1230, 1200, 1070, 1040, or 1100 nucleotides. More preferably, a first, second, third and/or further polynucleotide according to the present invention has a length of 420-2550, 420-2400, 420-2250, 420-2100, 420-1950, 420-1800, 420-1650, 420-1500, 420-1470, 420-1440, 420-1410, 420-1380, 420-1350, 420-1320, 420-1290, 420-1260, 420-1230, 420-1200, 420-1070, 420-1040, or 420-1100, 450-2550, 480-2550, 510-2550, 540-2550, 570-2550, 600-2550, 630-2550, 660-2550, 690-2550, 720-2550, 750-2550, 780-2550, 810-2550, 840-2550, 870-2550, 900-2550, 930-2550, 960-2550 or 990-2550 nucleotides

Preferably, a first and second polynucleotide according to the present invention each have a length of at least 420, 450, 480, 510, 540, 570, 600, 630, 660, 690, 720, 750, 780, 810, 840, 870, 900, 930, 960 or 990 nucleotides and/or a length of at most 2550, 2400, 2250, 2100, 1950, 1800, 1650, 1500, 1470, 1440, 1410, 1380, 1350, 1320, 1290, 1260, 1230, 1200, 1070, 1040, or 1100 nucleotides. More preferably, a first and second polynucleotide according to the present invention each have a length of 420-2550, 420-2400, 420-2250, 420-2100, 420-1950, 420-1800, 420-1650, 420-1500, 420-1470, 420-1440, 420-1410, 420-1380, 420-1350, 420-1320, 420-1290, 420-1260, 420-1230, 420-1200, 420-1070, 420-1040, or 420-1100, 450-2550, 480-2550, 510-2550, 540-2550, 570-2550, 600-2550, 630-2550, 660-2550, 690-2550, 720-2550, 750-2550,

780-2550, 810-2550, 840-2550, 870-2550, 900-2550, 930-2550, 960-2550 or 990-2550 nucleotides.

Preferably, a first, second and third polynucleotide according to the present invention each have a length of at least 420, 450, 480, 510, 540, 570, 600, 630, 660, 690, 720, 750, 780, 810, 840, 870, 900, 930, 960 or 990 nucleotides and/or a length of at most 2550, 2400, 2250, 2100, 1950, 1800, 1650, 1500, 1470, 1440, 1410, 1380, 1350, 1320, 1290, 1260, 1230, 1200, 1070, 1040, or 1100 nucleotides. More preferably, a first, second and third polynucleotide according to the present invention each have a length of 420-2550, 420-2400, 420-2250, 420-2100, 420-1950, 420-1800, 420-1650, 420-1500, 420-1470, 420-1440, 420-1410, 420-1380, 420-1350, 420-1320, 420-1290, 420-1260, 420-1230, 420-1200, 420-1070, 420-1040, or 420-1100, 450-2550, 480-2550, 510-2550, 540-2550, 570-2550, 600-2550, 630-2550, 660-2550, 690-2550, 720-2550, 750-2550, 780-2550, 810-2550, 840-2550, 870-2550, 900-2550, 930-2550, 960-2550 or 990-2550 nucleotides.

Preferably, a first, second, third and further polynucleotide according to the present invention each have a length of at least 420, 450, 480, 510, 540, 570, 600, 630, 660, 690, 720, 750, 780, 810, 840, 870, 900, 930, 960 or 990 nucleotides and/or a length of at most 2550, 2400, 2250, 2100, 1950, 1800, 1650, 1500, 1470, 1440, 1410, 1380, 1350, 1320, 1290, 1260, 1230, 1200, 1070, 1040, or 1100 nucleotides. More preferably, a first, second, third and further polynucleotide according to the present invention each have a length of 420-2550, 420-2400, 420-2250, 420-2100, 420-1950, 420-1800, 420-1650, 420-1500, 420-1470, 420-1440, 420-1410, 420-1380, 420-1350, 420-1320, 420-1290, 420-1260, 420-1230, 420-1200, 420-1070, 420-1040, or 420-1100, 450-2550, 480-2550, 510-2550, 540-2550, 570-2550, 600-2550, 630-2550, 660-2550, 690-2550, 720-2550, 750-2550, 780-2550, 810-2550, 840-2550, 870-2550, 900-2550, 930-2550, 960-2550 or 990-2550 nucleotides.

In a fourth aspect, the present invention provides for a polypeptide and/or a polynucleotide as defined herein above. Preferably, said polypeptide is any of the first, second, third and/or further polypeptide as defined in the second aspect of the invention. Preferably, said polynucleotide is encoding any of said first, second, third and/or further polypeptide. Preferably said polynucleotide is any of the first, second, third and/or further polynucleotide as defined in the third aspect of the invention. Preferably, said polypeptide comprises and/or consists of an enzymatic active domain,

a cell wall binding domain and optionally a tag for ease of purification as defined herein, preferably said enzymatic active domain being a cysteine, histidine-dependent amidohydrolases/peptidase domain, an endopeptidase domain or an amidase domain, and preferably said polypeptide comprises a multiplicity of said enzymatic active domain, preferably said multiplicity being 2, *i.e.* a duplicate. Preferably, said polypeptide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to any of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70 or 72. Preferably, said polynucleotide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to any of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69 or 71, or alternatively is able to hybridise under stringent conditions with these given sequences. More preferably, said polypeptide comprises and/or consists of a duplicated amidase domain and a cell wall binding domain and optionally a tag for ease of purification as defined herein, preferably said amidase domain is of *S. aureus* bacteriophage Φ2638a endolysin and said cell wall binding domain is of *S. Simulans* lysostaphin. Most preferably, said polypeptide comprises and/or consists of a duplicated endopeptidase domain and a cell wall binding domain and optionally a tag for ease of purification as defined herein, preferably said endopeptidase domain is a Peptidase_M23 domain of *S. Simulans* lysostaphin and said cell wall binding domain is of *S. Simulans* lysostaphin.

Preferably, said polypeptide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28, 34, 46, 52, 58 or 70, more preferably, said polypeptide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 28, 46, 52, or 70, even more preferably, said polypeptide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 46, or 70, most preferably said polypeptide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 70. Preferably said polynucleotide is encoding for any of the indicated preferred polypeptides.

Preferably, said polynucleotide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 27, 33, 45, 51, 57 or 69, or alternatively is able to hybridise under stringent conditions with these

given sequences. Preferably, said polypeptide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 27, 45, 51, or 69, or alternatively is able to hybridise under stringent conditions with these given sequences. Even more preferably, said polypeptide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 45, or 69, or alternatively is able to hybridise under stringent conditions with these given sequences. Most preferably said polypeptide has at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity with SEQ ID NO: 69, or alternatively is able to hybridise under stringent conditions with these given sequences.

In another embodiment, said polypeptide is not SEQ ID NO: 70 and/or said polynucleotide is not SEQ ID NO: 69.

In a fifth aspect the present invention relates to a combination according to the third aspect of the present invention and/or a polynucleotide of the fourth aspect, wherein a polynucleotide according to the third aspect and/or fourth aspect of the present invention is present in an expression construct. Moreover, said first, second and/or thirdand/or further polynucleotide can be present in an expression construct. Said expression construct may be 'naked' DNA or RNA preferably comprised in vesicles or liposomes, or may be comprised in an expression vector. Preferably said expression construct is an expression vector, more preferably a plasmid, a cosmid, a bacteriophage or a virus is transformed by introducing a polynucleotide encoding a first, second or third polypeptide as defined earlier herein and wherein said polynucleotide is operably linked to one or more control which direct the production or expression of the encoded polypeptide in a cell, a subject, or a cell-free system. Such transformation vectors according to the host organism to be transformed are well known to those skilled in the art and widely described in the literature. The expression construct may also be any DNA or RNA virus, such as, but not limited to, Adenovirus, Adeno-Associated Virus, Retrovirus, Lentivirus, Modified Vaccinia Nakara virus or Fowl Pox Virus or any other viral vector applicable of conferring expression of polypeptides into a chosen subject. DNA vectors may be non-integrating, such as episomally replicating vectors, or may be vectors integrating in the host genome by random integration or by homologous recombination. An expression vector may be seen as a recombinant expression vector.

Within the present invention, a first, second, third and/or fourth polynucleotide according to the present invention can be present on distinct expression constructs, or may be present combined on a single expression construct. Moreover, in a combination of the present invention said first polynucleotide can be present on a first expression construct while the second polynucleotide is present on a second expression construct. Furthermore, in a combination of the present invention, said first polynucleotide can be present on a first expression construct, the second on a second expression construct, the third present on a third expression construct, and the further present on a further expression construct. It is also encompassed within the present invention that the first, second, third and/or further are comprised on a single expression construct. Combinations are also possible, where two polypeptides of the present invention are present on a single expression construct, while one of them is present on a distinct expression construct.

In a sixth aspect, provided is a combination according to the third aspect of the invention and/or a polynucleotide of the fourth aspect,, wherein an expression construct according to the fifth aspect of the invention is present in an expression system. Within the present invention, said expression system may be a cell, preferably a microbial, prokaryotic or eukaryotic cell, which is suitable for expression of the polypeptide of the invention. In a preferred embodiment, said cell is an *E. coli*. In an even more preferred embodiment, said cell is *E. coli* XL1blue MRF`.

In a seventh aspect, provided is a process for the transformation of a host organism or a cell, by introducing at least one polynucleotide according to the present invention, which transformation may be carried out by any suitable known means which have been widely described in the specialist literature and in particular in the references cited in the present application, more particularly by the vector according to the present invention. Within the present invention, said first, second, third and/or further polypeptide as defined herein can be present in a single transformed host organism or cell or present in distinct transformed host organisms or cells. A cell may be any microbial, prokaryotic or eukaryotic cell, which is suitable for expression of the polypeptide of the present invention. In a preferred embodiment, said cell is an *E. coli*. In an even more preferred embodiment, said cell is *E. coli* XL1blue MRF'. A preferred method for producing, optionally purifying and optionally freeze-drying a first, second, third and/or further polypeptide as defined herein, comprises the steps of:

i) producing said first, second, third and/or further polypeptide in a cell comprising a expression construct as defined herein, optionally

ii) purifying said first, second, third and/or further polypeptide, and optionally

iii) freeze-drying said purified first, second, third and/or further polypeptide.

In a preferred embodiment, an *E. coli* is used in step i) for producing said first, second, third and/or further polypeptide using recombinant technologies. More preferably an *E. coli* XL1blueMRF is used in step i) for producing said first, second, third and/or further polypeptide using recombinant technologies. Preferably, in step ii), IMAC and Econo-Pac Chromatography columns (Biorad) packed with 5mL low density Nickel chelating agarose beads (ABT beads) in combination with gravity flow is used to purify said first, second, third and/or further polypeptide. The eluted first, second, third and/or further polypeptide can be dialyzed for 2, 4, and 12 hours against 3 x 1l lyophilization buffer, said buffer preferably comprising 50 mM phosphate, 500mM sucrose, 200mM mannitol, 0.005% polysorbate20, pH 7.4.

Even more preferred, said method for producing, optionally purifying and freeze-drying a first, second, third and/or further polypeptide as defined herein, further comprises a method of treating said first, second, third and/or further polypeptide obtainable by the method described above. Said treatment comprises substituting a divalent metal ion for increasing a lytic activity as compared to an untreated first, second, third and/or further polypeptide, preferably said method comprising the steps of:

iv) dialyzing said polypeptide against a buffer comprising a chelating compound;

v) dialyzing said polypeptide against a divalent metal ion-containing buffer, preferably said divalent metal ion being selected from the group consisting of $Mn^{2+}$, $Co^{2+}$, $Cu^{2+}$, and $Zn^{2+}$.

A "chelating compound" being defined herein as a compound that binds a metal ion. Well known chelating compounds are ethylene diamine tetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA). Preferably EDTA is used in step v) of the method described herein. Preferably, the divalent metal ion of step v) of said method is selected from the group consisting $Mn^{2+}$, $Co^{2+}$, $Cu^{2+}$, more preferably, said divalent metal ion is selected from the group consisting of $Mn^{2+}$ and $Co^{2+}$, even more preferably said divalent metal ion is $Mn^{2+}$.

In an eighth aspect of the present invention, a combination according to the first aspect of the present invention is present in at least two distinct compositions. Moreover, the invention provides a combination of the present invention, wherein a first composition comprises a source of first enzymatic active domain according to the present invention and a second composition comprises a source of second enzymatic active domain according to the present invention, wherein said first and second enzymatic active domains are comprised on distinct first and second polypeptides according to the first aspect, preferably wherein said first composition is free of a source of said second enzymatic domain and said second composition is free of a source of said first enzymatic active domain. In addition, the present invention provides a combination according to the present invention, wherein a first composition comprises a source of first enzymatic active domain according to the present invention, a second composition comprising a source of second enzymatic active domain according to the present invention, a third composition comprising a source of a third enzymatic active domain according to the present invention, and further composition comprising a source of a further enzymatic active domain according to the present invention, wherein said first, second, third and further enzymatic active domains are comprised on distinct first, second, third and further polypeptides according to the first aspect of the present invention, preferably wherein said first composition is free of said source of said second, third and further enzymatic domain, said second composition is free of said source of said first, third and further enzymatic active domain, said third composition is free of said source of said first, second and further enzymatic active domain and said further composition is free of said source of said first, second and third enzymatic active domain.

Moreover, the present invention provides a combination according to the present invention, wherein a first composition comprises a source of a first polypeptide according to the present invention, a second composition comprises a source of a second polypeptide according to the present invention, and optionally third and/or further composition comprises a source of a third and/or further respective polypeptide according to the present invention. Preferably said first composition is free of said source of a second polypeptide, free of said source of a third polypeptide and said free of source of a further respective polypeptide. Preferably, said second composition is free of said source of a first polypeptide, free of said source of a third polypeptide and

free of said source of a further respective polypeptide. Preferably said third composition is free of said source of a first polypeptide, free of said source of a second polypeptide and free of said source of a further respective polypeptide. Preferably said further composition is free of said source of a first polypeptide, free of said source of a second polypeptide and free of said source of a third polypeptide.

In a ninth aspect, the present invention provides a composition comprising any of the first, second, third and/or further polypeptide and/ or nucleotide of the fourth aspect of the invention. Preferably, the present invention provides for a composition comprising any of the first, second, third and/or further polypeptide as defined the second aspect of the invention and/or nucleotide as defined in the third aspect of the invention.

In a tenth aspect, the present invention provides a composition comprising a combination according to the first aspect of the present invention. Moreover, the invention provides a single composition comprising a source of a first enzymatic active domain according to the present invention and source of a second enzymatic active domain according to the present invention, wherein said first and second enzymatic active domains are comprised on distinct first and second polypeptides. Moreover, the present invention provides a composition according to the present invention comprising source of a first enzymatic active domain according to the present invention and a source of a second enzymatic active domain according to the present invention, wherein said first enzymatic active domain is comprised on a first polypeptide according to the first aspect of the present invention and said second enzymatic active domain is comprised on a second polypeptide according to the first aspect of the present invention, wherein said first polypeptide is free of said second enzymatic active domain and said second polypeptide is free of said first enzymatic active domain.

In addition, the invention provides a single composition comprising a source of a first enzymatic active domain according to the present invention, a source of a second enzymatic active domain according to the present invention, and a source of a third and/or further enzymatic active domain according to the present invention, wherein said first, second and third and/or further enzymatic active domains are comprised on distinct first, second and third and/or further polypeptides according to the first aspect of the present invention. Moreover, the present invention provides a composition comprising a source of a first enzymatic active domain according to the present

invention, a source of a second enzymatic active domain according to the present invention, and a source of a third and/or further enzymatic active domain according to the present invention wherein said first enzymatic active domain is comprised on a first polypeptide according to the first aspect of the present invention, said second enzymatic active domain is comprised on a second polypeptide according to the first aspect of the present invention, and said third and/or further enzymatic active domain is comprised on a third and/or further polypeptide according to the first aspect of the present invention, wherein said first polypeptide is free of said second enzymatic active domain and third and/or further enzymatic active domain, said second polypeptide is free of said first and third and/or further enzymatic active domain and said third and/or further polypeptide is free of said first and second enzymatic active domain.

The first, second and optionally third and/or further composition according to the eighth aspect of the present invention and/or a single composition according to the ninth and/or tenth aspect of the present invention may be in the liquid, solid or semi-liquid or semi-solid form. Preferably, a first, second and optionally third and/or further composition according to the eight aspect of the present invention and/or the single composition according to the ninth and/or tenth aspect of the present invention is an antimicrobial, preferably a food preservative or a disinfectant. Preferably said antimicrobial is for killing a bacterium, preferably a bacterium of the genus *Staphylococcus*, more preferably a bacterium of the species *Staphylococcus aureus*. Preferably, a first, second and optionally third and/or further composition according to the eight aspect of the present invention and/or a single composition according to the ninth and/or tenth aspect of the invention further comprises an pharmaceutical acceptable carrier and/or an additional active ingredient selected from the group consisting of a bacteriophage, a bacteriostatic agent, a bactericide agent, an antibiotic, a surfactant and/or an enzyme. An antibiotic of the present invention can be any antibiotic known in the art including antibiotics and chemotherapeutic agents, and including but not limited to vancomycin, nisin, danofloxacin and neomycin. An enzyme useful in a composition of the present invention includes but is not limited to enzymes that aid in breaking up biofims (e.g. biofilms found in food processing equipment) such as but not limited to polysaccharide depolymerise enzymes and protease. A surfactant useful in a composition of the present invention helps to wet the surface so that the active ingredient of the present invention, including the combination

of the present invention, is properly distributed over the various surfaces, and to solubilise and remove dirt so that the *Staphylococcus* are accessible to the active ingredients of the invention. Suitable surfactants include but are not limited to polysorbate (tween) 80, 20 and 81 and Dobanols (Shell Chemical Co. RTM).

An antimicrobial disinfectant composition of the present invention may further comprise surface disinfectants known in the art such as but not limited to benzoic acid and PBT, preferably disinfectants with which a combination of the first aspect of the present invention, preferably an expression system, even more preferably a (recombinant) bacteriophage of the present invention, is compatible.

The first, second and optionally third and/or further composition of the eight aspect of the present invention and/or the single composition of the ninth and/or tenth aspect of the present invention may further comprise a pharmaceutically acceptable carrier. Such composition is preferably for use as a medicine or as a medicament.

In a eleventh aspect, the present invention provides a combination of a first, second and optionally third and/or further composition according to the eight aspect of the present invention and/or a single composition according to the ninth and/or tenth aspect of the present invention for use as a medicament. Preferably said medicament is for the prevention or delay of a *Staphylococcus* related condition in a subject such as an infectious disease. More preferably, the invention relates to a pharmaceutical or medical composition for the treatment of a condition related to *Staphylococcus*. Preferably, the invention relates to a pharmaceutical or medical composition for the treatment of an infectious disease caused by a bacterium, preferably a bacterium of the genus *Staphylococcus*, more preferably a bacterium of the species *S. aureus*. Preferably, said infectious disease is a skin infection, mastitis, pneumonia, meningitis, endocarditis, Toxic Shock Syndrome (TSS), sepsis, septicemia, bacteremia, or osteomyelitis. Preferably, said skin infection is selected from the group of pimples, impetigo, boils, furuncles, cellulitis folliculitis, carbuncles, scaled skin syndrome and abscesses.

The combination of said first, second and optionally third and/or further composition according to the eight aspect of the present invention and/or the single composition according to the ninth and/or tenth aspect of the present invention is preferably said to be active, functional or therapeutically active or able to treat, prevent and/or delay an infectious disease when it decreases the amount of a Staphylococcus

genera present in a patient or in a cell of said patient or in a cell line or in a cell free in vitro system and preferably means that 99%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5% or less of the initial amount of a Staphylococcus genera, is still detectable. More preferably, no Staphylococcus genera is detectable. In this paragraph, the expression "amount of Staphylococcus genera" preferably means viable Staphylococci. Staphylococci of all genera may be detected using standard techniques known by the artisan such as immunohistochemical techniques using Staphylococcus specific antibodies, tube coagulase tests that detect staphylocoagulase or "free coagulase", detection of surface proteins such as clumping factor (slide coagulase test) and/or protein A (commercial latex tests). Viable Staphylococci may be detected using standard techniques known by the artisan such as microbiological bacterial culture techniques and/or real-time quantitative reverse transcription polymerase chain reaction to assay for bacterial mRNA.

A decrease according to the present invention is preferably assessed in a tissue or in a cell of an individual or a patient by comparison to the amount present in said individual or patient before treatment with said composition or polypeptide of the invention. Alternatively, the comparison can be made with a tissue or cell of said individual or patient which has not yet been treated with said composition or polypeptide in case the treatment is local.

Encompassed within the present invention is a method for treatment, prevention or delay of a microbial related condition in an individual, comprising administering to said individual a combination of a fist, second and optionally third and/or further composition according to the eighth aspect of the present invention. Moreover, the present invention provides the use of a combination according to the eight aspect of the present invention, wherein said first, second and optionally third and/or further enzymatic active domains are comprised on distinct first, second and optionally third and/or further polypeptides, wherein said first, second and optionally third and/or further polypeptides are comprised in separate compositions, for the manufacture of a medicament, preferably a medicament for the treatment, prevention or delay of a microbial related condition.

The combination of the first, second and optionally third and/or further composition according to the eighth aspect of the present invention and/or the single composition according to the ninth and/or tenth aspect of the present invention can be

used to treat animals, including humans, infected with *S. aureus*. Any suitable route of administration can be used to administer said combination of compositions or said single composition including but not limited to: oral, aerosol or other device for delivery to the lungs, nasal spray, intravenous, intramuscular, intraperitoneal, intrathecal, vaginal, rectal, topical, lumbar puncture, intrathecal, and direct application to the brain and/or meninges. A combination of composition or a single composition of the invention may be administered to a patient or of a cell, tissue or organ or said patient at least one week, one month, six month, one year or more. In an embodiment, said combination of compositions according to the seventh aspect of the present invention is administered separately. In an alternative embodiment, said combination is stored separately, and admixed just before administration. Preferably, said combination is admixed to comprise equimolar amounts of said first, second and optionally third and/or further polypeptide. Even more preferably, said combination is admixed to comprise equimolar amounts of said first, second and optionally third and/or further enzymatic active domain.

In a twelfth aspect, the present invention provides a use of a combination of compositions according to the eighth aspect or a single composition according to the ninth and/or tenth aspect of the present invention as an antimicrobial agent, preferably a food preservative or a disinfectant. An antimicrobial agent is for controlling a bacterium, preferably said bacterium is a Staphylococcus, more preferably said bacterium is *Staphylococcus aureus*. Preferably, an antimicrobial agent is for killing a bacterium, preferably said bacterium is a Staphylococcus, more preferably said bacterium is *Staphylococcus aureus*. A disinfectant is an antimicrobial agent specific for use on inanimate objects.

In a thirteenth aspect, the present invention provides a method for controlling microbial contamination in a food- or feed product, on and/or in food- or feed processing equipment or medical equipment on and/or in food- or feed containers comprising contacting a combination of compositions according to the eighth aspect of the present invention or a composition according the ninth and/or tenth aspect of the present invention with the food- or feed product, the food- or feed processing equipment or medical equipment and/or the food- or feed containers.

Preferably a method according to the present invention is for controlling bacterium of the genus *Staphylococcus*, more preferably a bacterium of the species

*Staphylococcus aureus*. Preferably, said method of controlling includes the reduction of counts of *Staphylococcus* bacteria and/or the prevention of their growth in the first place, in food products (including but not limited to the dairy industry) as well as in food processing plants in which the food products are being processed such as on processing equipment and other sites in food industry facilities, e.g. food storage container. Furthermore, said method of controlling includes the reduction of counts of *Staphylococcus* bacteria and/or the prevention of their growth in the first place, in medical equipment. Preferably, said method is of controlling for cleaning and sterilizing medical equipement, such as fiberscopes, like gastrocameras, peritoneoscopes, thoracoscopes and arthoroscopes, and medical supplies, like catheters and tubes that have long ducts or hollow portions and tend to be repetitively employed by being introduced into human bodies.

A method of the present invention encompasses the application of a combination of compositions according to the eight aspect of the present invention and/or a composition according to the ninth and/or tenth aspect of the present invention on or into food products, and/or into various physical sites within the food processing plants on or in food processing equipment, by a number of means including, but not limited to, admixing, spraying or directly applying said combination of compositions of the eight aspect or composition of the ninth and/or tenth aspect of the present invention.

In a further embodiment, a combination of a source of said first aspect of the present invention can be isolated from said source, wherein said source is an expression system, such as a recombinant cell or a recombinant bacteriophage can be directly applied or administered without isolation of said polypeptide. For example, a cell which produces a first and second and optionally a third and/or further polypeptide of the present invention could be administered to a subject (human or animal) or applied to a surface where said first and second and optionally said third and/or further polypeptide of the present invention would be secreted into food, onto a surface or into the subject's gut. The combination of the present invention can then bind and optionally lyse bacterial cells, preferably a bacterium of the genus *Staphylococcus*, more preferably a bacterium of the species *Staphylococcus aureus*, present in this environment. The applications as defined herein significantly reduce the numbers of *Staphylococcus* bacteria that would otherwise be present.

Optionally, the method of the present invention can be combined with any sterilization method or disinfectant known in the art such as ultrasonic cleaning, irradiation or thermal sterilization, by immersing the equipment in a disinfectant solution such as ethanol, ammonium, iodine and/or aldehyde disinfectant, or by using gas sterilization by retaining the device in a closed atmosphere such as formaline gas or ethylene oxide gas.

Definitions

"Sequence identity" or “identity” in the context of amino acid- or nucleic acid-sequence is herein defined as a relationship between two or more amino acid (peptide, polypeptide, or protein) sequences or two or more nucleic acid (nucleotide, polynucleotide) sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between amino acid or nucleotide sequences, as the case may be, as determined by the match between strings of such sequences. Within the present invention, sequence identity with a particular sequence preferably means sequence identity over the entire length of said particular polypeptide or polynucleotide sequence. The sequence information as provided herein should not be so narrowly construed as to require inclusion of erroneously identified bases. The skilled person is capable of identifying such erroneously identified bases and knows how to correct for such errors.

"Similarity" between two amino acid sequences is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one peptide or polypeptide to the sequence of a second peptide or polypeptide. In a preferred embodiment, identity or similarity is calculated over the whole SEQ ID NO as identified herein. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heine, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include e.g. the GCG program package (Devereux, J., et al., Nucleic Acids Research 12 (1): 387 (1984)), BestFit, BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Mol. Biol. 215:403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215:403-410 (1990). The well-known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992); Gap Penalty: 12; and Gap Length Penalty: 4. A program useful with these parameters is publicly available as the "Ogap" program from Genetics Computer Group, located in Madison, WI. The aforementioned parameters are the default parameters for amino acid comparisons (along with no penalty for end gaps).

Preferred parameters for nucleic acid comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0; Gap Penalty: 50; Gap Length Penalty: 3. Available as the Gap program from Genetics Computer Group, located in Madison, Wis. Given above are the default parameters for nucleic acid comparisons.

Optionally, in determining the degree of amino acid similarity, the skilled person may also take into account so-called "conservative" amino acid substitutions, as will be clear to the skilled person. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulphur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution

groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Substitutional variants of the amino acid sequence disclosed herein are those in which at least one residue in the disclosed sequences has been removed and a different residue inserted in its place. Preferably, the amino acid change is conservative. Preferred conservative substitutions for each of the naturally occurring amino acids are as follows: Ala to ser; Arg to lys; Asn to gln or his; Asp to glu; Cys to ser or ala; Gln to asn; Glu to asp; Gly to pro; His to asn or gln; Ile to leu or val; Leu to ile or val; Lys to arg; gln or glu; Met to leu or ile; Phe to met, leu or tyr; Ser to thr; Thr to ser; Trp to tyr; Tyr to trp or phe; and, Val to ile or leu.

A polynucleotide is represented by a nucleotide sequence. A polypeptide is represented by an amino acid sequence. A nucleic acid construct is defined as a polynucleotide which is isolated from a naturally occurring gene or which has been modified to contain segments of polynucleotides which are combined or juxtaposed in a manner which would not otherwise exist in nature. Optionally, a polynucleotide present in a nucleic acid construct is operably linked to one or more control sequences, which direct the production or expression of said peptide or polypeptide in a cell or in a subject.

As used herein the term "heterologous sequence" or "heterologous nucleic acid" is one that is not naturally found operably linked as neighboring sequence of said first nucleotide sequence. As used herein, the term “heterologous” may mean “recombinant”. "Recombinant" refers to a genetic entity distinct from that generally found in nature. As applied to a nucleotide sequence or nucleic acid molecule, this means that said nucleotide sequence or nucleic acid molecule is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in the production of a construct that is distinct from a sequence or molecule found in nature.

“Operably linked” is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the nucleotide sequence coding for the polypeptide of the invention such that the control sequence directs the production/expression of the peptide or polypeptide of the invention in a cell and/or in a subject.

“Operably linked” may also be used for defining a configuration in which a sequence is appropriately placed at a position relative to another sequence coding for a

functional domain such that a chimeric polypeptide is encoded in a cell and/or in a subject.

Expression will be understood to include any step involved in the production of the peptide or polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification and secretion.

Optionally, a promoter represented by a nucleotide sequence present in a nucleic acid construct is operably linked to another nucleotide sequence encoding a peptide or polypeptide as identified herein.

The term "transformation" refers to a permanent or transient genetic change induced in a cell following the incorporation of new DNA (*i.e.* DNA exogenous to the cell). When the cell is a bacterial cell, as is intended in the current invention, the term usually refers to an extrachromosomal, self-replicating vector which harbors a selectable antibiotic resistance.

An expression vector may be any vector which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of a nucleotide sequence encoding a polypeptide of the invention in a cell and/or in a subject. As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more genes or nucleic acids, located upstream with respect to the direction of transcription of the transcription initiation site of the gene. It is related to the binding site identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites, and any other DNA sequences, including, but not limited to, transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one skilled in the art to act directly or indirectly to regulate the amount of transcription from the promoter. Within the context of the invention, a promoter preferably ends at nucleotide -1 of the transcription start site (TSS).

"Polypeptide" as used herein refers to any peptide, oligopeptide, polypeptide, gene product, expression product, or protein. A polypeptide is comprised of consecutive amino acids. The term "polypeptide" encompasses naturally occurring or synthetic molecules.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of a polypeptide. Each control sequence may be native or foreign to the nucleic acid sequence encoding the

polypeptide. Such control sequences include, but are not limited to, a leader, optimal translation initiation sequences (as described in Kozak, 1991, J. Biol. Chem. 266:19867-19870), a polyadenylation sequence, a pro-peptide sequence, a pre-pro-peptide sequence, a promoter, a signal sequence, and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals.

The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

The control sequence may be an appropriate promoter sequence, a nucleic acid sequence, which is recognized by a host cell for expression of the nucleic acid sequence. The promoter sequence contains transcriptional control sequences, which mediate the expression of the polypeptide. The promoter may be any nucleic acid sequence, which shows transcriptional activity in the cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the cell.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator, which is functional in the cell, may be used in the present invention.

The control sequence may also be a suitable leader sequence, a non-translated region of an mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence, which is functional in the cell, may be used in the present invention.

The control sequence may also be a polyadenylation sequence, a sequence which is operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence, which is functional in the cell, may be used in the present invention.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but

items not specifically mentioned are not excluded. In addition the verb “to consist” may be replaced by “to consist essentially of” meaning that a product or a composition or a nucleic acid molecule or a peptide or polypeptide of a nucleic acid construct or vector or cell as defined herein may comprise additional component(s) than the ones specifically identified; said additional component(s) not altering the unique characteristic of the invention. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the elements is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Description of the figures

**Figure 1**: target bond sites of cysteine, histidine-dependent amidohydrolases/peptidase (CHAP) domain, an endopeptidase domain (Peptidase_M23), an amidase domain (Ami2638), a muramidase domain and a glycosaminidase domain

**Figure 2**: SDS PAGE of partially purified proteins. 1: HXaM23-LST_CWT-LST (SEQ ID NO: 46), 2: HXaM23-LST_CWT-NM3 (SEQ ID NO: 48), 3: HXaM23-LST_CBD2638 (SEQ ID NO: 44), 4: HXaCHAPTw_CWT-NM3 (SEQ ID NO: 42), 5: HXaCHAPTw_CWT-LST (SEQ ID NO: 40), 6: HXaCHAPTw_CBD2638 (SEQ ID NO: 38), 7: HXaCHAPK_CWT-NM3 (SEQ ID NO: 36), 8: HXaCHAPK_CWT-LST (SEQ ID NO: 34), 9: HXaCHAPK_CBD2638 (SEQ ID NO: 32), 10: HXaAmi2638_CWT-NM3 (SEQ ID NO: 30), 11: HXaAmi2638_CWT-LST (SEQ ID NO: 28), 12: HXaAmi2638_CBD2638 (SEQ ID NO: 26).

**Figure 3**: SDS PAGE of partially purified proteins. 1: HXaCHAPK_CBD2638 (SEQ ID NO: 32), 2: HXaCHAPK_CWT-LST (SEQ ID NO: 34), 3: HXaCHAPK_CWT-NM3 (SEQ ID NO: 36), 4: HXaCHAPK_CHAPK_CBD2638 (SEQ ID NO: 56), 5: HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58), 6: HXaCHAPK_CHAPK_CWT-NM3 (SEQ ID NO: 60).

**Figure 4**: SDS PAGE of partially purified proteins. 1: HXaAmi23638_Ami2638_CBD2638 (SEQ ID NO: 50), 2: HXaAmi23638_Ami2638_CWT-LST (SEQ ID NO: 52), 3: HXaAmi23638_Ami2638_CWT-NM3 (SEQ ID NO: 54), 4: HXaM23-LST_M23-LST_CBD2638 (SEQ ID NO: 68), 5: HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 5:** Effect of CHAPK containing lysins at 50 nM and 200 nM protein assay concentration against *S. aureus* BB270 cells. Tested constructs: HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58) and HXaCHAPK_CHAPK_CBD2638 (SEQ ID NO: 56)

**Figure 6:** Effect of M23-LST containing lysins at 50 nM and 200 nM protein assay concentration against *S. aureus* BB270 cells. Tested constructs: HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70), HXaM23-LST_M23-LST_ CBD2638 (SEQ ID NO: 68) and HXaM23-LST_CWT-LST (SEQ ID NO: 46)

**Figure 7:** Effect of Ami2638 containing lysins at 50 nM and 200 nM protein assay concentration against *S. aureus* BB270 cells. Tested constructs: HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52), HXaAmi2638_Ami2638_CBD2638 (SEQ ID NO: 50), HXaAmi2638_CWT-LST (SEQ ID NO: 28),, HXaAmi2638_CBD2638 (SEQ ID NO: 26)

**Figure 8:** Comparison of 50 nM protein mixtures (16.67 nM each protein) with equal CBDs. Tested constructs: HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58), HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52), HXaCHAPK_CHAPK_CBD2638 (SEQ ID NO: 56), HXaM23-LST_M23-LST_ CBD2638 (SEQ ID NO: 68), HXaAmi2638_Ami2638_CBD2638 (SEQ ID NO: 50), HXaCHAPK_CBD2638 (SEQ ID NO: 32), HXaM23-LST_CBD2638(SEQ ID NO: 44) and HXaAmi2638_CBD2638 (SEQ ID NO: 26).

**Figure 9:** Comparison of 200 nM protein mixtures (66.67 nM each protein) with equal CBDs. Tested constructs: HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58), HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52), HXaCHAPK_CHAPK_CBD2638 (SEQ ID NO: 56), HXaM23-LST_M23-LST_ CBD2638 (SEQ ID NO: 68), HXaAmi2638_Ami2638_CBD2638 (SEQ ID NO: 50), HXaCHAPK_CBD2638 (SEQ ID NO: 32), HXaM23-LST_CBD2638(SEQ ID NO: 44) and HXaAmi2638_CBD2638 (SEQ ID NO: 26).

**Figure 10:** Comparison of protein mixtures of HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58), HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70) and HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52) at (16.67 nM and 66.67 nM of

each protein) with 50 nM and 200 nM of the reference protein M23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 11:** Effect of CHAPK, M23 and Ami containing lysins at 30 nM protein assay concentration against *S. aureus* BB270 cells. Tested constructs: HXaAmi2638_CWT-LST (SEQ ID NO: 28), HXaCHAPK_CWT-LST (SEQ ID NO: 34), HXaM23-LST_CWT-LST (SEQ ID NO: 46), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52), HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58) and HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 12:** Effect of 30nM protein mixtures (15nM each protein). Tested constructs: HXaAmi2638_CWT-LST (SEQ ID NO: 28), HXaCHAPK_CWT-LST (SEQ ID NO: 34), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52) and HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58).

**Figure 13:** Effect of 30nM protein mixtures (15nM each protein). Tested constructs: HXaAmi2638_CWT-LST (SEQ ID NO: 28), HXaM23-LST_CWT-LST (SEQ ID NO: 46), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52) and HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 14:** Effect of 30nM protein mixtures (15nM each protein). Tested constructs: HXaCHAPK_CWT-LST (SEQ ID NO: 34), HXaM23-LST_CWT-LST (SEQ ID NO: 46), HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58) and HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 15:** Effect of 30nM protein mixtures (10 nM each protein). Tested constructs: HXaAmi2638_CWT-LST (SEQ ID NO: 28), HXaCHAPK_CWT-LST (SEQ ID NO: 34), HXaM23-LST_CWT-LST (SEQ ID NO: 46), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52), HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58) and HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 16:** Effect of CHAPK, M23 and Ami containing lysins at 50 nM protein assay concentration against *S. aureus* BB270 cells. Tested constructs: HXaAmi2638_CWT-LST (SEQ ID NO: 28), HXaCHAPK_CWT-LST (SEQ ID NO: 34), HXaM23-LST_CWT-LST (SEQ ID NO: 46), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52), HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58) and HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 17:** Effect of 50nM protein mixtures (25nM each protein). Tested constructs: HXaAmi2638_CWT-LST (SEQ ID NO: 28), HXaCHAPK_CWT-LST (SEQ ID NO: 34), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52) and HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58).

**Figure 18:** Effect of 50nM protein mixtures (25nM each protein). Tested constructs: HXaAmi2638_CWT-LST (SEQ ID NO: 28), HXaM23-LST_CWT-LST (SEQ ID NO: 46), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52) and HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 19:** Effect of 50nM protein mixtures (25nM each protein). Tested constructs: HXaCHAPK_CWT-LST (SEQ ID NO: 34), HXaM23-LST_CWT-LST (SEQ ID NO: 46), HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58) and HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

**Figure 20:** Effect of 50nM protein mixtures (16.67 nM each protein). Tested constructs: HXaAmi2638_CWT-LST (SEQ ID NO: 28), HXaCHAPK_CWT-LST (SEQ ID NO: 34), HXaM23-LST_CWT-LST (SEQ ID NO: 46), HXaAmi2638_Ami2638_CWT-LST (SEQ ID NO: 52), HXaCHAPK_CHAPK_CWT-LST (SEQ ID NO: 58) and HXaM23-LST_M23-LST_CWT-LST (SEQ ID NO: 70).

Examples

Example 1

**MATERIALS AND METHODS**

**Bacteria, phages, and plasmids**

Bacterial strains for cloning and protein production, phages, and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue MRF` (Stratagene, La Jolla, CA, U.S.) and *E. coli* Sure (Stratagene) served for cloning and over-expression of N-terminal 6xHis-tagged recombinant fusion proteins. Constructs containing repetitive sequences were processed in *E. coli* Sure strain. *E. coli* was cultured in Luria-Bertani (LB) medium at 37°C supplemented with 100 µg/ml ampicillin and 30 µg/ml tetracycline for cloning, and at 30°C with 100 µg/ml ampicillin for plasmid selection during protein expression. *Staphylococcus aureus*, BB270 NCTC8325*mec* used as substrate in lysis assays, was grown in , half concentrated Brain Heart Infusion medium (BHI, Biolife, Milano, Italy) at 37°C. Log phase cells from two liter cultures were harvested, PBST (50 mM $NaH_2PO_4$, 120 mM NaCl, 0.1% Tween 20, pH 7.4) washed, 100 fold concentrated and aliquots thereof were stored at -80°C.

**DNA techniques and cloning procedures**

Cloning and construction of fusion proteins were performed using standard techniques (Loessner *et al.* Mol Microbiol 2002, 44: 335-349; Sambrook *et al.* 1989 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York). Enzymes were purchased from New England Biolabs (Ipswitch, MA, U.S.), Fermentas (Burlington, Canada , Roche Basel, Switzerland) and Qiagen. Endolysins and separated enzymatically active domains (EAD) coding regions from phages Φ2638a, Φ187, ΦK, and ΦTwort were in frame amplified from purified phage DNA or phage lysate. Plasmid DNA served as template for amplification of EAD encoding gene fragments of lysostaphin with High Fidelity PCR Enzyme Mix (Fermentas). Restriction sites for insert ligation into pQE-30 protein expression plasmid (Qiagen) and its derivatives were introduced by the primers. Plasmids constructed or used in this study are listed in Table 1. Protein expression plasmids were transformed into electro-competent *E. coli* XL1BlueMRF` and Plasmids containing repetitive sequences into electro-competent *E. coli* Sure. DNA concentrations were determined with a spectrophotometer (NanoDrop

ND-1000 Spectrophotometer, Thermo scientific, Waltham, MA, U.S.). Sequence integrities were confirmed by nucleotide sequencing (GATC, Konstanz, Germany). Constructs bearing a single N-terminal enzymatically active domain (EAD) and a C-terminal cell wall binding domain (CBD), or a cell wall targeting domain (CWT) respectively were constructed by creating in-frame fusions of the respective coding regions with splicing overlap extension PCR (SOE). These fragments were inserted into SacI – SalI restriction sites of pQE-30Xa vector DNA. On the basis of these vectors, constructs with repetitive duplicated EADs were obtained by introducing the respective EAD coding sequences into StuI – SacI sites. For full construction principles please refer to Table 1.

**Expression and purification of recombinant fusion-proteins**

Protein overexpression and immobilized metal affinity chromatography (IMAC) purification of N-terminal 6xHis tagged proteins was done with minor modifications as previously described by others (Loessner *et al.* Appl Environ Microbiol 1996, 62: 3057-3060; Schmelcher *et al.* Appl Environ Microbiol 2010, 76: 5745-5756; Eichenseher *et al.*, unpublished). Briefly, heterologous proteins expression was induced by the addition of 0.2-0.5 mM IPTG to log phase *E. coli* cultures, grown in LB medium at 30°C. Cells were further incubated at the same temperature before harvesting by centrifugation. *E. coli* were lysed in immobilization buffer (50 mM $NaH_2PO_4$, 500 mM NaCl, 5 mM imidazole, 0.1% Tween 20, pH 7.4) by a double passage through a French Pressure Cell Press (1200 psi, SLM Aminco, Urbana, IL, U.S.) operated at 1200 psi. Insoluble cell debris was removed by centrifugation and filter sterilization (0.2 µm PES membrane, Millipore) prior to gravity flow IMAC purification in MicroBiospin (Bio-Rad, Hercules, CA, U.S.) columns packed with low density Ni-NTA Superflow resin (Chemie Brunschwig AG, Basel, Switzerland). After column washing, 6xHis tagged proteins were eluted using elution buffer (50 mM $NaH_2PO_4$, 500 mM NaCl, 125 mM imidazole, 0.1% Tween 20, pH 7.4) and dialyzed against dialysis buffer (50 mM $NaH_2PO_4$, 100 mM NaCl, 0.1% Tween 20, pH 7.4). CHAP homologues domain containing proteins were subjected to buffer exchange using EconoPak 10DG columns (Biorad) using CHAP buffer (50 mM Tris, 5 mM $CaCl_2$, 10% glycerol, pH 7.4). Protein purities were estimated by SDS-PAGE and concentrations were determined spectrophotometrically (NanoDrop ND-1000 spectrophotmeter) or with a Pierce BCA

Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, U.S.) according to the manufacturer`s manual. Proteins were stored in 50% glycerol at -20°C.

**Photometric determination of lysis kinetics**

Lytic activities were measured in turbidity reduction assays using a Wallac VICTOR$^3$ TM14200 (Perkin Elmer, Waltham, MA, U.S.) multilable counter device. Substrate cells from frozen stock were washed with buffer and adjusted to an optical density at 595nm ($OD_{595nm}$) of 1 +/- 0.05 using Macro Cuvettes (Greiner Bio-one, Kremsmünster, Austria) and a spectrophotometer (BioChrom, Cambridge, UK). *Staphylococcus* lytic enzymes were diluted with buffer to equimolar quantities and if desired, subsequently pooled to obtain enzyme mixtures. 10 µl protein solutions were distributed in crystal grade multi-well polystyrene tissue culture test plates (SPL Lifesciences, Poncheon-Si, Korea) and mixed with 190 µl substrate cell suspension using a multichannel pipette. Reduction in turbidity over the time was monitored at $OD_{595nm}$ with vigorous plate shaking in between the reads. As a control to monitor autolytic activity under the given conditions served 10 µl buffer or water. Assays were performed in triplicates. Calculation of relative activity values were obtained as described elsewhere (Korndorfer *et al.* J Mol Biol 2006, 364: 678-689; Schmelcher *et al.*, Microb Biotechnol. 2011, 4(5): 651-662). Sigmoidal lysis- and control curves were normalized to a common starting value of 1.

**RESULTS**

Downstream processing of cytosolic expressed *Staphylococcus* lytic proteins resulted in soluble proteins with purities depending on the protein structure and origin. The majority of the constructs had by SDS-PAGE estimated purities of up to >90% (Figures 2-4).

We tested a selection of the partially purified proteins in turbidity reduction assays (lysis assays). Individual lysins and combinations thereof were tested against *S. aureus* BB270 cells from frozen stock in PBST buffer at pH 7.4 and at different protein concentrations. CHAPK_CHAPK_CWT-LST (SEQ ID NO: 58, encoded by SEQ ID NO: 47) and CHAPK_CHAPK_CBD2638 (SEQ ID NO: 56 encoded by SEQ ID NO: 55) proteins were virtually inactive at 50nM assay concentrations against a cell

suspension set to an optical density at 595nm (OD595nm) of ~1, but displayed significant activities at 200 nM assay concentrations (Figure 5).

Using M23-LST (SEQ ID NO: 16, encoded by SEQ ID NO: 15) containing proteins in an identical assay setup, best results were achieved using M23-LST_M23-LST_CWT-LST (SEQ ID NO: 70, encoded by SEQ ID NO: 69) at 200 nM assay concentration. The CWT-LST (SEQ ID NO: 4, encoded by SEQ ID NO: 3) appears to be superior to CBD2638 (SEQ ID NO: 6, encoded by SEQ ID NO: 5). Furthermore, repetitive double M23-LST variants (SEQ ID NO: 68 and 70 encoded by SEQ ID NO: 67 and 69, respectively) were found superior to single M23-LST (SEQ ID NO: 44 and 46, encoded by SEQ ID NO: 43 and 45, respectively). This effect was found more pronounced at 50nM protein concentrations. For full results please refer to figure 6.

All lysins built with Ami2638 (SEQ ID NO: 18, encoded by SEQ ID NO: 17) were significantly less active compared to CHAPK (SEQ ID NO: 10, encoded by SEQ ID NO: 9) and M23-LST (SEQ ID NO: 16, encoded by SEQ ID NO:15) proteins. Here, CBD2638 (SEQ ID NO: 6 encoded by SEQ ID NO: 5) was superior to CWT-LST (SEQ ID NO: 4, encoded by SEQ ID NO: 3). Duplication of the catalytic domain had little effect when combined with CBD2638 (SEQ ID NO: 6, encoded by SEQ ID NO: 5), but duplication added a positive effect on lysis kinetics when combined with CWT-LST (SEQ ID NO: 4, encoded by SEQ ID NO: 3) (figure 7).

We also compared activities of mixtures of proteins built with CWT-LST (SEQ ID NO: 4, encoded by SEQ ID NO: 3) or CBD2638 (SEQ ID NO: 6, encoded by SEQ ID NO: 5). At low protein concentrations (16.67 nM each, or 50nM total protein concentration respectively), mixtures of CWT-LST (SEQ ID NO: 58, 70 and 52) proteins were found significantly more active than mixtures of CBD2638 (SEQ ID NO: 56, 68 and 50) proteins. Furthermore, duplication of the EADs had little effect on lysis kinetics in CBD2638 constructs mixtures (SEQ ID NO: 56, 68 and 50 as compared to SEQ ID NO: 32, 44 and 26) (figure 8). Increasing the assay concentration of proteins to 200 nM (66.67 nM each) resulted in virtually equal activities of CWT-LST and CBD2638 constructs with repetitive doubled EADs. Although it appears that the lysis curve of CBD2638 constructs (figure 9) runs “above” the curve of CWT-LST constructs, we estimate lysis kinetics being equal. This is simply because assays were performed in 96 well plates and OD595nm measurements started not at the same time points after lysine addition. The first measurement of the curve was already at a stage were lysis

commenced, so normalization of the curve to an initial OD595nm of 1 shifted the curve to higher values. Unlike at 50 nM protein concentrations, mixtures of CBD2638 constructs with only single EAD were not found equally active as repetitive doubled EAD-CBD2638 constructs, but showed slower lysis kinetics.

Finally, we compared the most effective mixture consisting of Ami2638_Ami2638_CWT-LST (SEQ ID NO: 52, encoded by SEQ ID NO: 51), CHAPK_CHAPK_CWT-LST (SEQ ID NO: 58, encoded by SEQ ID NO: 57), and M23-LST_M23-LST_CWT-LST (SEQ ID NO: 70, encoded by SEQ ID NO: 69) with the most effective reference protein M23-LST_M23-LST_CWT-LST (SEQ ID NO: 70, encoded by SEQ ID NO: 69). At both concentrations tested (50 nM and 200 nM total protein concentrations), the mixtures were found slightly superior to M23-LST_M23-LST_CWT-LST (SEQ ID NO: 70, encoded by SEQ ID NO: 69) (figure 10).

Example 2

MATERIAL AND METHODS

The lysis kinetics of single and combinations/mixtures of protein constructs produced according to Example 1 have been tested using the turbidity reduction assay as described in the Material and Method section of Example 1.

RESULTS

Lysis curves of the proteins and mixtures are shown in Figure 11 to 20. From these cures, maximum measured activity of each protein or mixture was calculated using 5-parameter sigmoidal fit model with SigmaPlot software. The first derivative of the slope is the maximum drop in optical density (OD595nm) and is defined as maximum measured activity. Table 3 is a summary table of the maximum measured activity of each protein or mixture.

**Table 1:** Bacterial strains, bacteriophages and plasmids

| Strain, phage, or plasmid | Genotype or relevant characteristics | Source or reference |
|---|---|---|
| Bacterial strains | | |
| *E. coli* XL-1BlueMRF` | Δ mcrA 183 Δ mcrCB-hsdSMR-mrr 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F` proAB laclq ZΔM15 Tn10 $Tet^r$ ] | Stratagene |
| *E. coli* Sure | e14- McrA- Δ mcrCB-hsdSMR-mrr 171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 $Kan^r$ uvrC [F′ proAB laclqZΔM15 Tn10 $Tet^r$ ] | Stratagene |
| Phages | | |
| *S. aureus* Φ187 | Siphoviridae | Loessner *et al.* J Bacteriol 1999, 181: 4452-4460. |
| *S. aureus* Φ2638 | Siphoviridae | Kwan *et al.* Proc Natl Acad Sci U S A 2005, 102: 5174-5179. |
| *S. aureus* ΦK | Siphoviridae | O'Flaherty *et al.* J Bacteriol 2005, 187: 7161-7164. |
| *S. aureus* ΦTw | Siphoviridae | Loessner *et al.* FEMS Microbiol Lett 1998, 162: 265-274. |
| Plasmids | | |
| pQE-30Xa | 3.4 kb cloning and expression vector; T5 promoter; creates N-terminal fusion of gene product with 21-aminoacid leader containing a 6x His-tag and Factor Xa protease cleavage site; $Amp^r$ | Qiagen |

| | | |
|---|---|---|
| pHXaAm2638_CBD2638 | *ply*2638 fragment encoding Ala143 – Lys486 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaAm2638_CWT-LST | In-frame fusions of *ply*2638 fragment encoding Ala143 – Asp392 and *pre- pro- lysostaphin* encoding Trp402 – Lys493 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaAm2638_CWT-NM3 | In-frame fusions of *ply*2638 fragment encoding Ala143 – Asp392 and *ply187* encoding Gly158 – Phe251 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaCHAPK_CBD2638 | In-frame fusions of *lysK* fragment encoding Met1 – Ala165 and *ply2638* encoding Gly360 – Lys486 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaCHAPK_CWT-LST | In-frame fusions of *lysK* fragment encoding Met1 – Ala165 and *pre-pro-lysostaphin* encoding Gly388 – Lys493 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaCHAPK_CWT-NM3 | In-frame fusions of *lysK* fragment encoding Met1 – Ala165 and *ply187* encoding Gly158 – Phe251 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaCHAPTw_CBD2638 | In-frame fusions of *PlyTw* fragment encoding Met1 – Asn182 and *ply2638* encoding Trp393 – Lys486 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaCHAPTw_CWT-LST | In-frame fusions of *PlyTw* fragment encoding Met1 – Asn182 and *pre-pro-lysostaphin* encoding Trp402 – Lys493 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaCHAPTw_CWT-NM3 | In-frame fusions of *PlyTw* fragment encoding Met1 – Ala165 and *ply187* encoding Gly158 – Phe251 cloned into SacI - SalI sites of pQE-30Xa | This study |

| | | |
|---|---|---|
| pHXaM23-LST_CBD2638 | In-frame fusions of *pre- pro- lysostaphin* fragment encoding Ala251 – Pro398 and *ply2638* encoding Trp393 – Lys486 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaM23-LST_CWT-LST | *pre- pro- lysostaphin* fragment encoding Ala251 - Lys493 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaM23-LST_CWT-NM3 | In-frame fusions of *pre- pro- lysostaphin* fragment encoding Ala251 – Gly401 and *ply187* encoding Gly158 – Phe251 cloned into SacI - SalI sites of pQE-30Xa | This study |
| pHXaAm2638_Am2638_CBD2638 | *ply*2638 fragment encoding Ala143 – Gly359 cloned into StuI - SacI sites of pHXaAm2638_CBD2638 | This study |
| pHXaAm2638_Am2638_CWT-LST | *ply*2638 fragment encoding Ala143 – Gly359 cloned into StuI - SacI sites of pHXaAm2638_CWT-LST | This study |
| pHXaAm2638_Am2638_CWT-NM3 | *ply*2638 fragment encoding Ala143 – Gly359 cloned into StuI - SacI sites of pHXaAm2638_CWT-NM3 | This study |
| pHXaCHAPK_CHAPK_CBD2638 | *lysK* fragment encoding Met1 – Asn195 cloned into StuI - SacI sites of pHXaCHAPK_CBD2638 | This study |
| pHXaCHAPK_CHAPK_CWT-LST | *lysK* fragment encoding Met1 – Asn195 cloned into StuI - SacI sites of pHXaCHAPK_CWT-LST | This study |
| pHXaCHAPK_CHAPK_CWT-NM3 | lysK fragment encoding Met1 – Asn195 cloned into StuI - SacI sites of pHXaCHAPK_CWT-NM3 | This study |
| pHXaCHAPTw_CHAPTw_CBD2638 | *plyTw* fragment encoding Met1 – Asn182 cloned into StuI - SacI sites of pHXaCHAPTw_CBD2638 | This study |
| pHXaCHAPTw_CHAPTw_CWT-LST | *plyTw* fragment encoding Met1 – Asn182 cloned into StuI - SacI sites of pHXaCHAPTw_CWT-LST | This study |

| | | |
|---|---|---|
| pHXaCHAPTw_CHAPTw_CWT-NM3 | *plyTw* fragment encoding Met1 – Asn182 cloned into StuI - SacI sites of pHXaCHAPTw_CWT-NM3 | This study |
| pHXaM23-LST_M23-LST_CBD2638 | *pre- pro- lysostaphin* fragment encoding Ala251 – Gly401 cloned into StuI - SacI sites of pHXaM23-LST_CBD2638 | Donovan *et al.* FEMS Microbiol Lett 2006, 265: 133-139. |
| pHXaM23-LST_M23-LST_CWT-LST | *pre- pro- lysostaphin* fragment encoding Ala251 – Gly401 cloned into StuI - SacI sites of pHXaM23-LST_CWT-LST | This study |
| pHXaM23-LST_M23-LST_CWT-NM3 | *pre- pro- lysostaphin* fragment encoding Ala251 – Gly401 cloned into StuI - SacI sites of pHXaM23-LST_CWT-NM3 | This study |

**Table 2:** SEQ ID NO overview table

| SEQ ID NO | Name construct | organism |
|---|---|---|
| 1 | Ply2638 endolysin CDS | Bacteriophage 2638A |
| 2 | Ply2638 endolysin PRT | Bacteriophage 2638A |
| 3 | CWT-LST CDS | *S.* simulans |
| 4 | CWT-LST PRT | *S.* simulans |
| 5 | CBD2638 CDS | Bacteriophage 2638A |
| 6 | CBD2638 PRT | Bacteriophage 2638A |
| 7 | CWT-NM3 CDS | *S. aureus* phage phiNM3 |
| 8 | CWT-NM3 PRT | *S. aureus* phage phiNM3 |
| 9 | CHAPK CDS | *S.* phage K |
| 10 | CHAPK PRT | *S.* phage K |
| 11 | CHAP-ΦTwort CDS | S. phage Twort |
| 12 | CHAP-ΦTwort PRT | S. phage Twort |
| 13 | M23-2638 CDS | Bacteriophage 2638A |
| 14 | M23-2638 PRT | Bacteriophage 2638A |
| 15 | M23-LST CDS | *S. simulans* |
| 16 | M23-LST PRT | *S. simulans* |
| 17 | Ami2638 CDS | Bacteriophage 2638A |
| 18 | Ami2638 PRT | Bacteriophage 2638A |
| 19 | CHAPK_CHAPK_CWT-LST CDS | artificial construct |
| 20 | CHAPK_CHAPK_CWT-LST PRT | artificial construct |
| 21 | M23-LST_M23-LST_CWT-LST CDS | artificial construct |
| 22 | M23-LST_M23-LST_CWT-LST PRT | artificial construct |
| 23 | Ami2638_ami2638_CWT-LST CDS | artificial construct |
| 24 | Ami2638_ami2638_CWT-LST PRT | artificial construct |
| 25 | HXaAmi2638_CBD2638 CDS | artificial construct |
| 26 | HXaAmi2638_CBD2638 PRT | artificial construct |
| 27 | HXaAmi2638_CWT-LST CDS | artificial construct |
| 28 | HXaAmi2638_CWT-LST PRT | artificial construct |
| 29 | HXaAmi2638_CWT-NM3 CDS | artificial construct |
| 30 | HXaAmi2638_CWT-NM3 PRT | artificial construct |
| 31 | HXaCHAPK_CBD2638 CDS | artificial construct |
| 32 | HXaCHAPK_CBD2638 PRT | artificial construct |
| 33 | HXaCHAPK_CWT-LST CDS | artificial construct |
| 34 | HXaCHAPK_CWT-LST PRT | artificial construct |
| 35 | HXaCHAPK_CWT-NM3 CDS | artificial construct |
| 36 | HXaCHAPK_CWT-NM3 PRT | artificial construct |
| 37 | HXaCHAPTw_CBD2638 CDS | artificial construct |

| | | |
|---|---|---|
| 38 | HXaCHAPTw_CBD2638 PRT | artificial construct |
| 39 | HXaCHAPTw_CWT-LST CDS | artificial construct |
| 40 | HXaCHAPTw_CWT-LST PRT | artificial construct |
| 41 | HXaCHAPTw_CWT-NM3 CDS | artificial construct |
| 42 | HXaCHAPTw_CWT-NM3 PRT | artificial construct |
| 43 | HXaM23-LST_CBD2638 CDS | artificial construct |
| 44 | HXaM23-LST_CBD2638 PRT | artificial construct |
| 45 | HXaM23-LST_CWT-LST CDS | artificial construct |
| 46 | HXaM23-LST_CWT-LST PRT | artificial construct |
| 47 | HXaM23-LST_CWT-NM3 CDS | artificial construct |
| 48 | HXaM23-LST_CWT-NM3 PRT | artificial construct |
| 49 | HXaAmi2638_Ami2638_CBD2638 CDS | artificial construct |
| 50 | HXaAmi2638_Ami2638_CBD2638 PRT | artificial construct |
| 51 | HXaAmi2638_Ami2638_CWT-LST CDS | artificial construct |
| 52 | HXaAmi2638_Ami2638_CWT-LST PRT | artificial construct |
| 53 | HXaAmi2638_Ami2638_CWT-NM3 CDS | artificial construct |
| 54 | HXaAmi2638_Ami2638_CWT-NM3 PRT | artificial construct |
| 55 | HXaCHAPK_CHAPK_CBD2638 CDS | artificial construct |
| 56 | HXaCHAPK_CHAPK_CBD2638 PRT | artificial construct |
| 57 | HXaCHAPK_CHAPK_CWT-LST CDS | artificial construct |
| 58 | HXaCHAPK_CHAPK_CWT-LST PRT | artificial construct |
| 59 | HXaCHAPK_CHAPK_CWT-NM3 CDS | artificial construct |
| 60 | HXaCHAPK_CHAPK_CWT-NM3 PRT | artificial construct |
| 61 | HXaCHAPTw_CHAPTw_CBD2638 CDS | artificial construct |
| 62 | HXaCHAPTw_CHAPTw_CBD2638 PRT | artificial construct |
| 63 | HXaCHAPTw_CHAPTw_CWT-LST CDS | artificial construct |
| 64 | HXaCHAPTw_CHAPTw_CWT-LST PRT | artificial construct |
| 65 | HXaCHAPTw_CHAPTw_CWT-NM3 CDS | artificial construct |
| 66 | HXaCHAPTw_CHAPTw_CWT-NM3 PRT | artificial construct |
| 67 | HXaM23-LST_M23-LST_CBD2638 CDS | artificial construct |
| 68 | HXaM23-LST_M23-LST_CBD2638 PRT | artificial construct |
| 69 | HXaM23-LST_M23-LST_CWT-LST CDS | artificial construct |
| 70 | HXaM23-LST_M23-LST_CWT-LST PRT | artificial construct |
| 71 | HXaM23-LST_M23-LST_CWT-NM3 CDS | artificial construct |
| 72 | HXaM23-LST_M23-LST_CWT-NM3 PRT | artificial construct |
| 73 | His-tag with linker CDS | artificial construct |
| 74 | His-tag with linker PRT | artificial construct |

**Table 3:** Results of Example 2.

**Single protein constructs (30 nM protein concentration)**

| protein construct | SEQ ID NO | mean ($\Delta_{max}OD_{595nm}*min^{-1}$) | std.dev. | std.err. |
|---|---|---|---|---|
| HXaAmi2638_CWT-LST | 28 | -0.004345 | 0.000722 | 0.000511 |
| HXaCHAPK_CWT-LST | 34 | -0.005163 | 0.000552 | 0.000390 |
| HXaM23-LST_CWT-LST | 46 | -0.013310 | 0.000387 | 0.000224 |
| HXaAmi2638_Ami2638_CWT-LST | 52 | -0.006686 | 0.000462 | 0.000267 |
| HXaCHAPK_CHAPK_CWT-LST | 58 | -0.004086 | 0.000304 | 0.000176 |
| HXaM23-LST_M23-LST_CWT-LST | 70 | -0.040422 | 0.000624 | 0.000360 |

**Mixtures of two protein constructs (30 nM total, 15 nM each protein)**

| protein construct | SEQ ID NO | mean ($\Delta_{max}OD_{595nm}*min^{-1}$) | std.dev. | std.err. |
|---|---|---|---|---|
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CWT-LST | 28 + 34 | -0.004492 | 0.000222 | 0.000157 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST | 52 + 58 | -0.010524 | 0.002527 | 0.001459 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CWT-LST | 52 + 34 | -0.004471 | 0.000125 | 0.000072 |
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST | 28 + 58 | -0.006872 | 0.000850 | 0.000491 |
| HXaAmi2638_CWT-LST<br>HXaM23-LST_CWT-LST | 28 + 46 | -0.018363 | 0.000199 | 0.000115 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 52 + 70 | -0.060616 | 0.004117 | 0.002377 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaM23-LST_CWT-LST | 52 + 46 | -0.020094 | 0.001989 | 0.001148 |
| HXaAmi2638_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 28 + 70 | -0.049715 | 0.005762 | 0.003327 |
| HXaCHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 34 + 46 | -0.009839 | 0.000700 | 0.000404 |
| HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 58 + 70 | -0.039957 | 0.001111 | 0.000641 |
| HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 58 + 46 | -0.011577 | 0.003904 | 0.002254 |
| HXaCHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 34 + 70 | -0.029355 | 0.000913 | 0.000527 |

**Mixtures of three protein constructs (30 nM total, 10 nM each protein)**

| protein construct | SEQ ID NO | mean ($\Delta_{max}OD_{595nm}*min^{-1}$) | std.dev. | std.err. |
|---|---|---|---|---|
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 28 + 34 + 46 | -0.013973 | 0.001444 | 0.000834 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 52 + 58 + 70 | -0.052270 | 0.007606 | 0.004391 |
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 28 + 58 + 70 | -0.045011 | 0.003443 | 0.001988 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 52 + 34 + 70 | -0.042337 | 0.003308 | 0.001910 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 52 + 58 + 46 | -0.020569 | 0.003307 | 0.001910 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 52 + 34 + 46 | -0.016268 | 0.000576 | 0.000333 |
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 28 + 58 + 46 | -0.013975 | 0.000365 | 0.000211 |
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 28 + 34 + 70 | -0.036804 | 0.003481 | 0.002010 |

**Single protein constructs (50 nM proteinconcentration)**

| protein construct | SEQ ID NO | mean ($\Delta_{max}OD_{595nm}*min^{-1}$) | std.dev. | std.err. |
|---|---|---|---|---|
| HXaAmi2638_CWT-LST | 28 | -0.005109 | 0.000061 | 0.000035 |
| HXaCHAPK_CWT-LST | 34 | -0.004037 | 0.000369 | 0.000261 |
| HXaM23-LST_CWT-LST | 46 | -0.022770 | 0.000304 | 0.000175 |
| HXaAmi2638_Ami2638_CWT-LST | 52 | -0.008042 | 0.000593 | 0.000342 |
| HXaCHAPK_CHAPK_CWT-LST | 58 | -0.003674 | 0.000121 | 0.000086 |
| HXaM23-LST_M23-LST_CWT-LST | 70 | -0.054314 | 0.000820 | 0.000474 |

**Mixtures of two protein constructs (50 nM total, 25 nM each protein)**

| protein construct | SEQ ID NO | mean ($\Delta_{max}OD_{595nm}$*$min^{-1}$) | std.dev. | std.err. |
|---|---|---|---|---|
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CWT-LST | 28 + 34 | -0.004611 | 0.000945 | 0.000668 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST | 52 + 58 | -0.007071 | 0.000287 | 0.000203 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CWT-LST | 52 + 34 | -0.006845 | 0.000578 | 0.000334 |
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST | 28 + 58 | -0.005107 | 0.000756 | 0.000436 |
| HXaAmi2638_CWT-LST<br>HXaM23-LST_CWT-LST | 28 + 46 | -0.029038 | 0.000591 | 0.000341 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 52 + 70 | -0.077677 | 0.005683 | 0.003281 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaM23-LST_CWT-LST | 52 + 46 | -0.033351 | 0.001808 | 0.001044 |
| HXaAmi2638_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 28 + 70 | -0.076113 | 0.000463 | 0.000268 |
| HXaCHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 34 + 46 | -0.014407 | 0.000917 | 0.000529 |
| HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 58 + 70 | -0.048809 | 0.000527 | 0.000304 |
| HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 58 + 46 | -0.018130 | 0.001014 | 0.000586 |
| HXaCHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 34 + 70 | -0.046676 | 0.002135 | 0.001233 |

**Mixtures of three protein constructs (50 nM total, 16.67 nM each protein)**

| protein construct | SEQ ID NO | mean ($\Delta_{max}OD_{595nm}*min^{-1}$) | std.dev. | std.err. |
|---|---|---|---|---|
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 28 + 34 + 46 | -0.020491 | 0.001630 | 0.000941 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 52 + 58 + 70 | -0.062127 | 0.002998 | 0.001731 |
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 28 + 58 + 70 | -0.054493 | 0.002078 | 0.001200 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 52 + 34 + 70 | -0.054908 | 0.000584 | 0.000337 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 52 + 58 + 46 | -0.025062 | 0.000831 | 0.000480 |
| HXaAmi2638_Ami2638_CWT-LST<br>HXaCHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 52 + 34 + 46 | -0.023737 | 0.000656 | 0.000379 |
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CHAPK_CWT-LST<br>HXaM23-LST_CWT-LST | 28 + 58 + 46 | -0.018786 | 0.000215 | 0.000124 |
| HXaAmi2638_CWT-LST<br>HXaCHAPK_CWT-LST<br>HXaM23-LST_M23-LST_CWT-LST | 28 + 34 + 70 | -0.051336 | 0.000409 | 0.000236 |

Claims

1. A combination of a source of a first enzymatic active domain and a source of a second enzymatic active domain, wherein said first and second enzymatic active domains each exhibit distinct target bond specificities and are comprised on a distinct first and second polypeptide.

2. A combination according to claim 1, wherein said different target bonds are essential bonds in a peptidoglycan layer of a bacterial cell, preferably wherein said bacterial cell is a *Staphylococcus*.

3. A combination according to claim 2, wherein said first and/or said second enzymatic active domain is a domain selected from the group consisting of a cysteine, histidine-dependent amidohydrolases/peptidase domain, an endopeptidase domain, an amidase domain and a glycosylhydrolase.

4. A combination according to claim 1-3, wherein said first and second polypeptide comprise a different multiplicity of said first and/or second enzymatic active domain.

5. A combination according to claim 1-4, wherein each of said distinct first and second polypeptide further comprises a cell wall-binding domain.

6. A combination according to claim 5, wherein:

- said first enzymatic active domain is a cysteine, histidine-dependent amidohydrolases/peptidase domain and said second enzymatic active domain is an endopeptidase domain,
- said combination further comprises a source of a third enzymatic active domain comprised on a distinct third polypeptide,
- said third enzymatic active domain is an amidase domain,
- said distinct third polypeptide further comprises a cell wall-binding domain, and
- each of said distinct first, second and third polypeptide comprises a multiplicity of said first, second and third enzymatic active domain.

7. A combination according to claim 6, wherein said first polypeptide comprises a sequence that has at least 80% sequence identity to SEQ ID NO: 58, said second polypeptide comprises a sequence that has at least 80% sequence identity SEQ ID NO: 70 and said third polypeptide comprises a sequence that has at least 80% sequence identity SEQ ID NO: 52.

8. A combination according to any of the claims 1-7, wherein said source of a first enzymatic active domain comprises a polypeptide and/or said source of a second enzymatic active domain comprises a polypeptide.

9. A combination according to any of the claims 1-7, wherein said source of a first enzymatic active domain comprises a polynucleotide encoding said first enzymatic active domain and/or said source of a second enzymatic active domain comprises a polynucleotide encoding said second enzymatic active domain.

10. A combination according to claim 9, wherein said polynucleotide encoding said first enzymatic active domain is present in an expression construct and/or said polynucleotide encoding said second enzymatic active domain is present in an expression construct, preferably wherein said expression construct is present in an expression system.

11. A composition comprising a combination according to any of the claims 1-10.

12. A composition according to claim 11, further comprising an pharmaceutical acceptable carrier and/or an additional active ingredient selected from the group consisting of a bacteriophage, a bacteriostatic agent, a bactericide agent, an antibiotic, a surfactant and/or an enzyme.

13. A composition according to claim 11 or 12 for use as a medicament, preferably for the treatment, prevention or delay of a *Staphylococcus* related condition in a subject.

14. Use of a composition according to claim 11 or 12 as an antimicrobial agent, preferably a food preservative or a disinfectant.

15. A method for controlling microbial contamination in a food- or feed product, on and/or in food- or feed processing equipment or medical equipment, on and/or in food- or feed containers, comprising contacting a composition according to claim 11 or 12 with the food- or feed product, the food- or feed processing equipment or medical equipment and/or the food- or feed containers.

# Fig 1

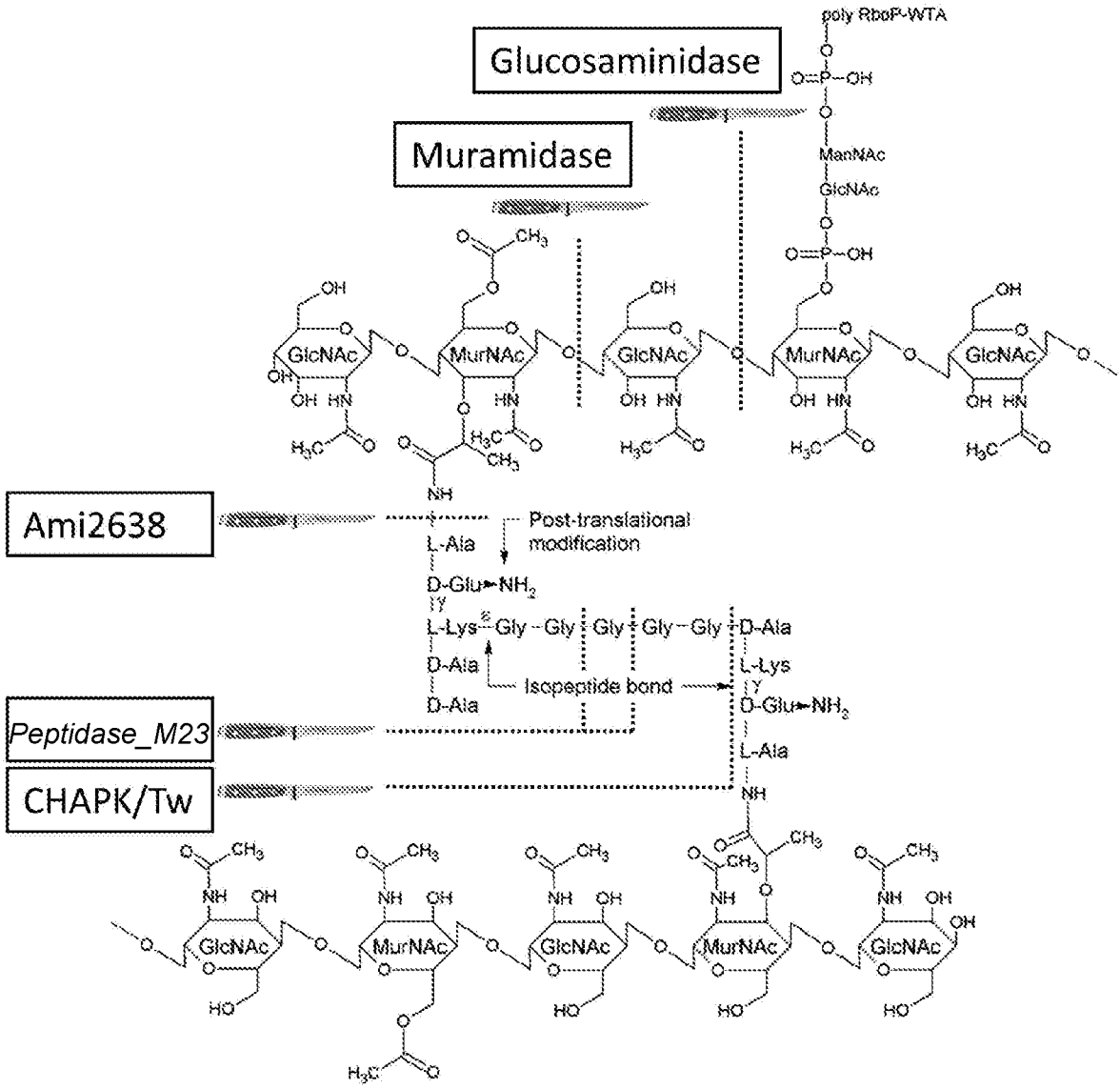

poly RboP-WTA
Glucosaminidase
Muramidase
ManNAc
GlcNAc
GlcNAc
MurNAc
GlcNAc
MurNAc
GlcNAc
Ami2638
L-Ala
Post-translational modification
D-Glu-NH2
L-Lys
Gly
Gly
Gly
Gly
Gly
D-Ala
D-Ala
Isopeptide bond
L-Lys
D-Glu-NH2
D-Ala
Peptidase_M23
CHAPK/Tw
GlcNAc
MurNAc
GlcNAc
MurNAc
GlcNAc

Fig 2

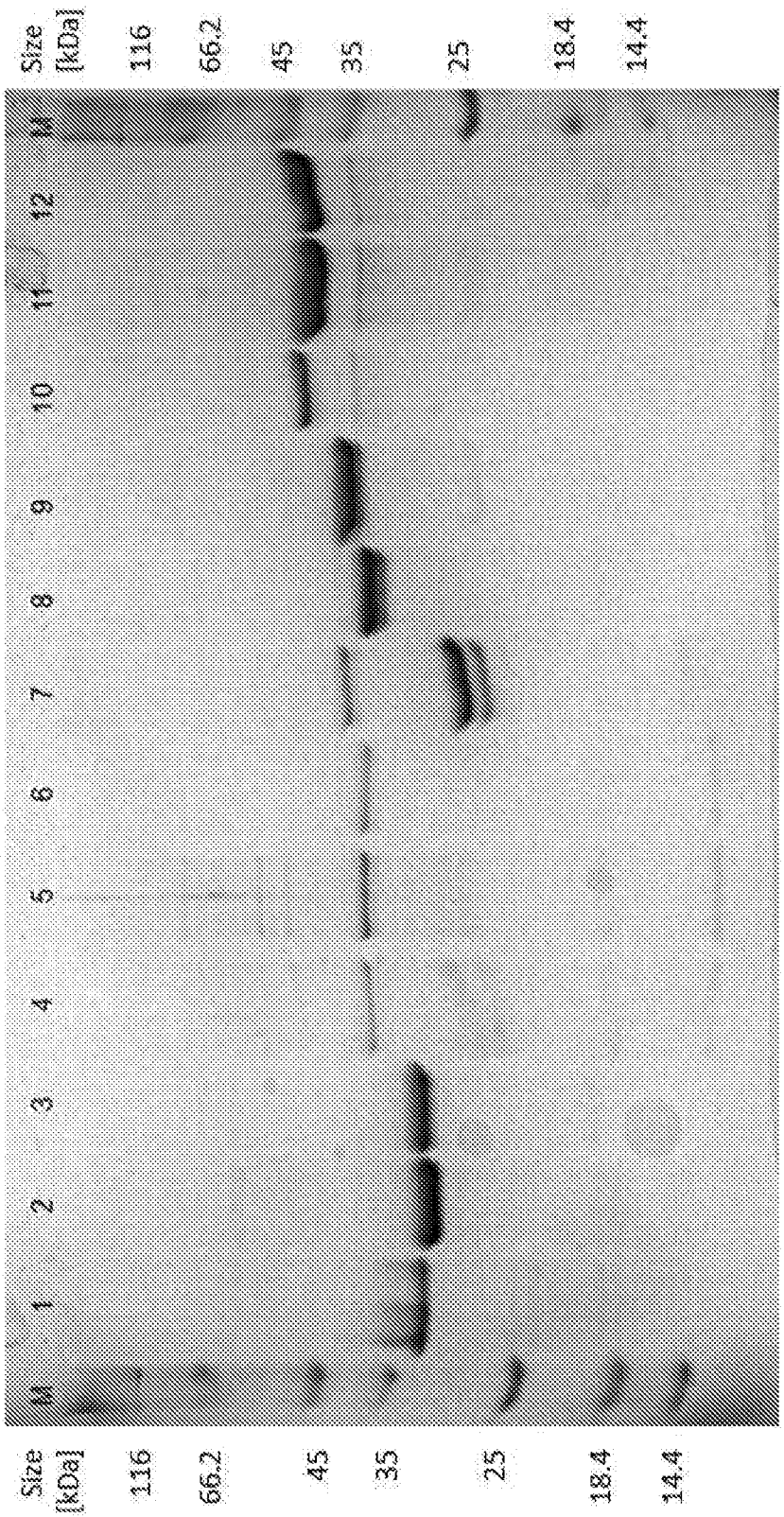


Fig 3

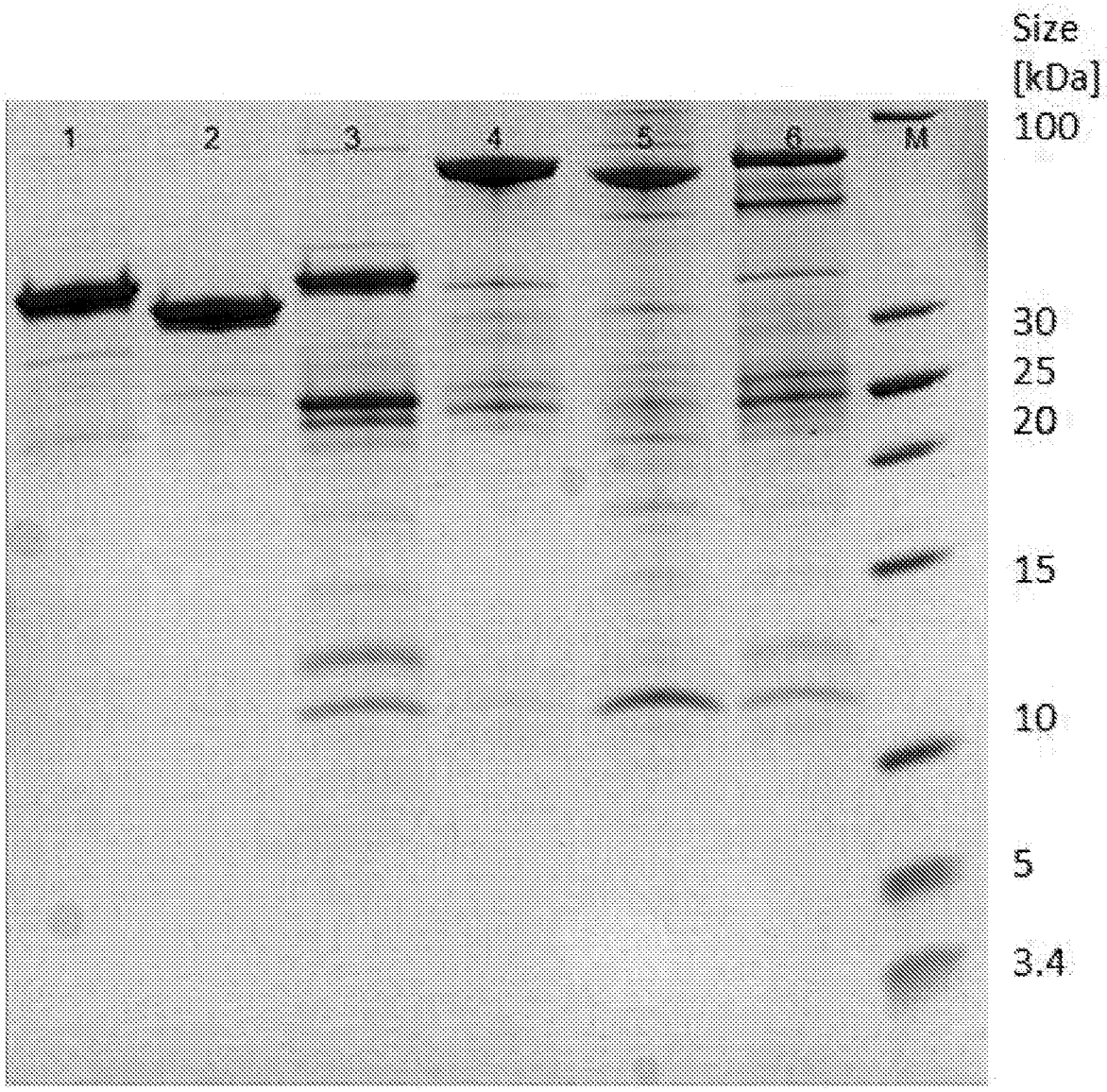


Fig 4

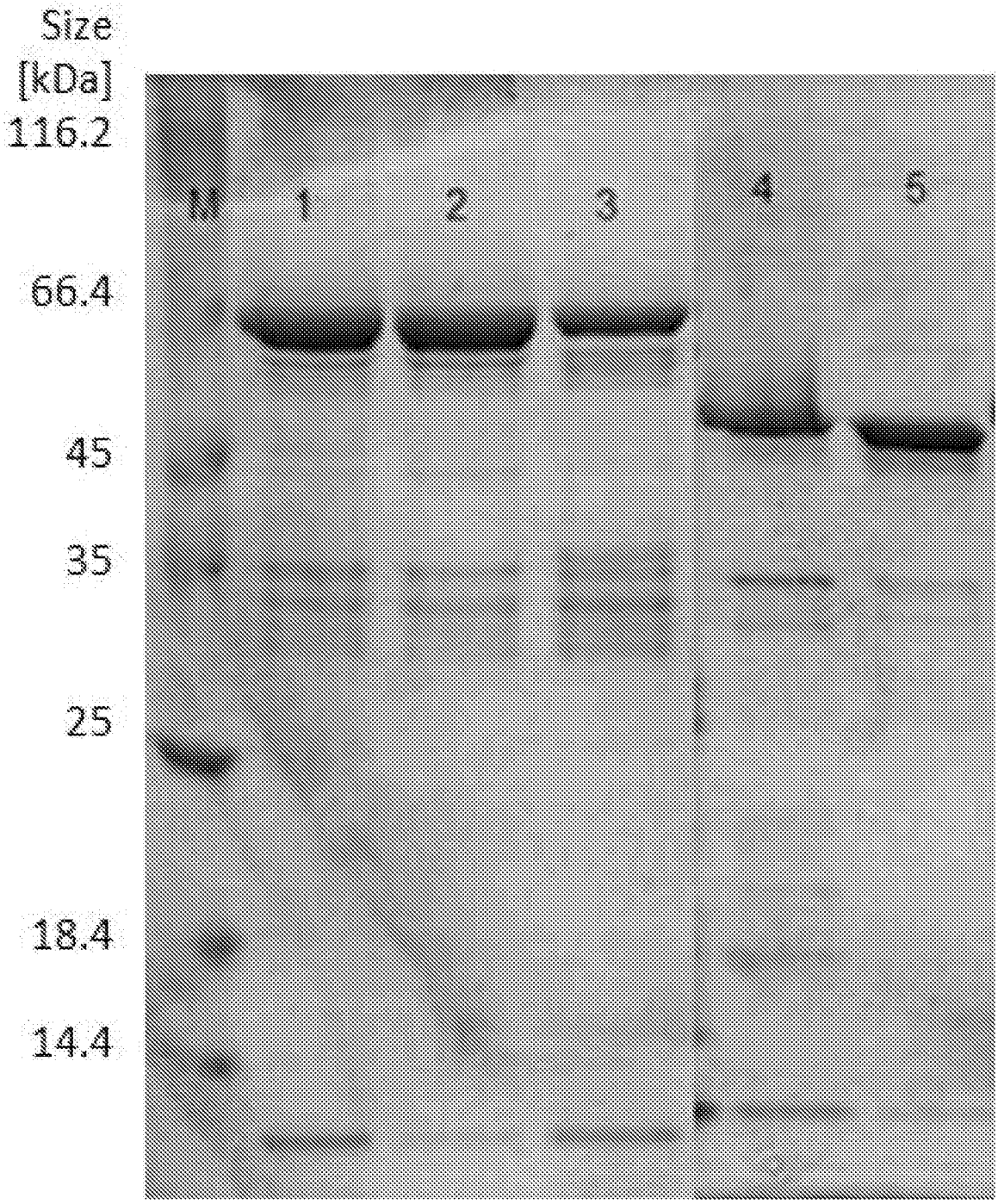
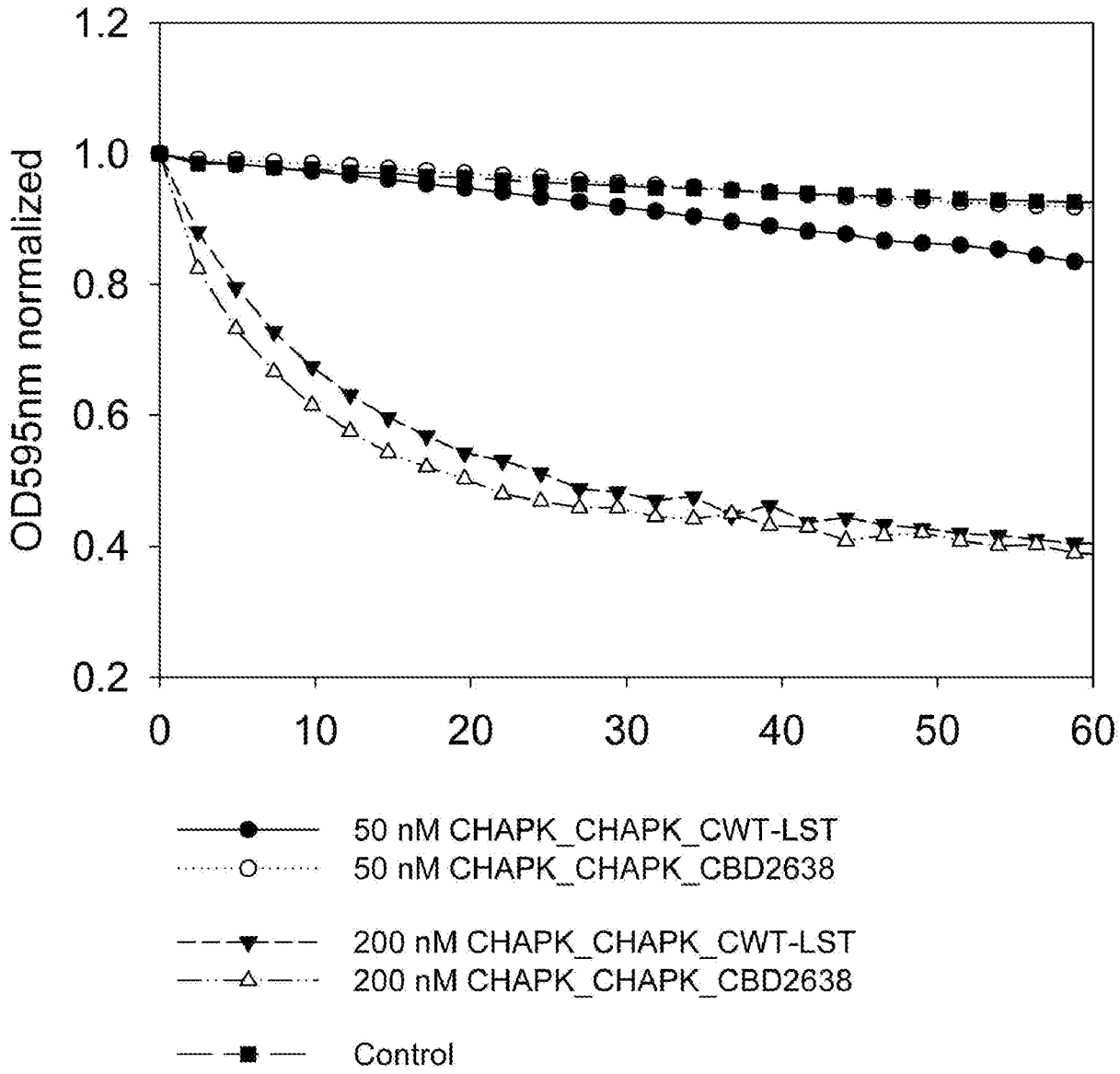


*Fig 5*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0.8
0.6
0.4
0.2
OD595nm normalized
0
10
20
30
40
50
60
50 nM CHAPK_CHAPK_CWT-LST
50 nM CHAPK_CHAPK_CBD2638
200 nM CHAPK_CHAPK_CWT-LST
200 nM CHAPK_CHAPK_CBD2638
Control

*Fig 6*

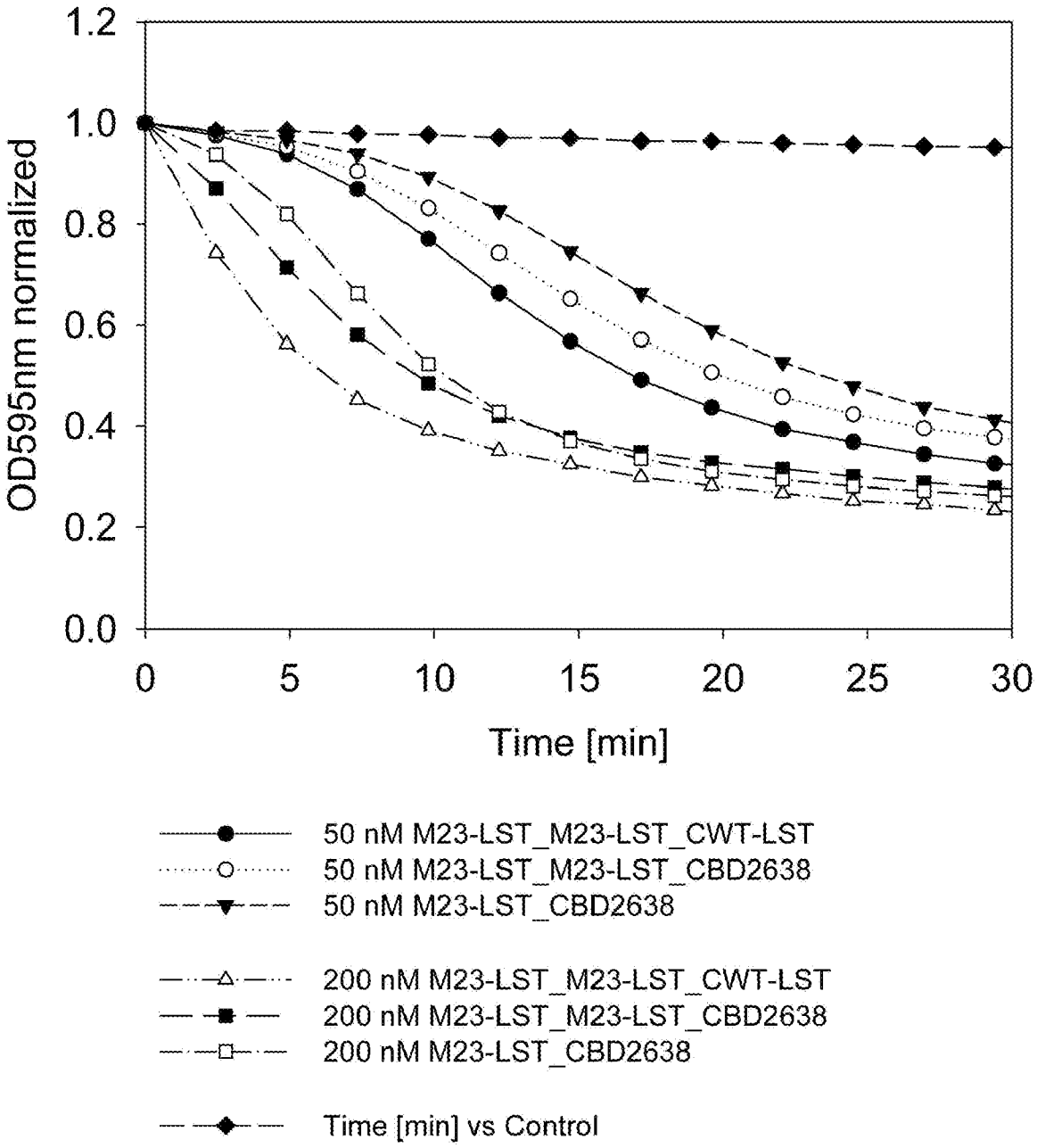
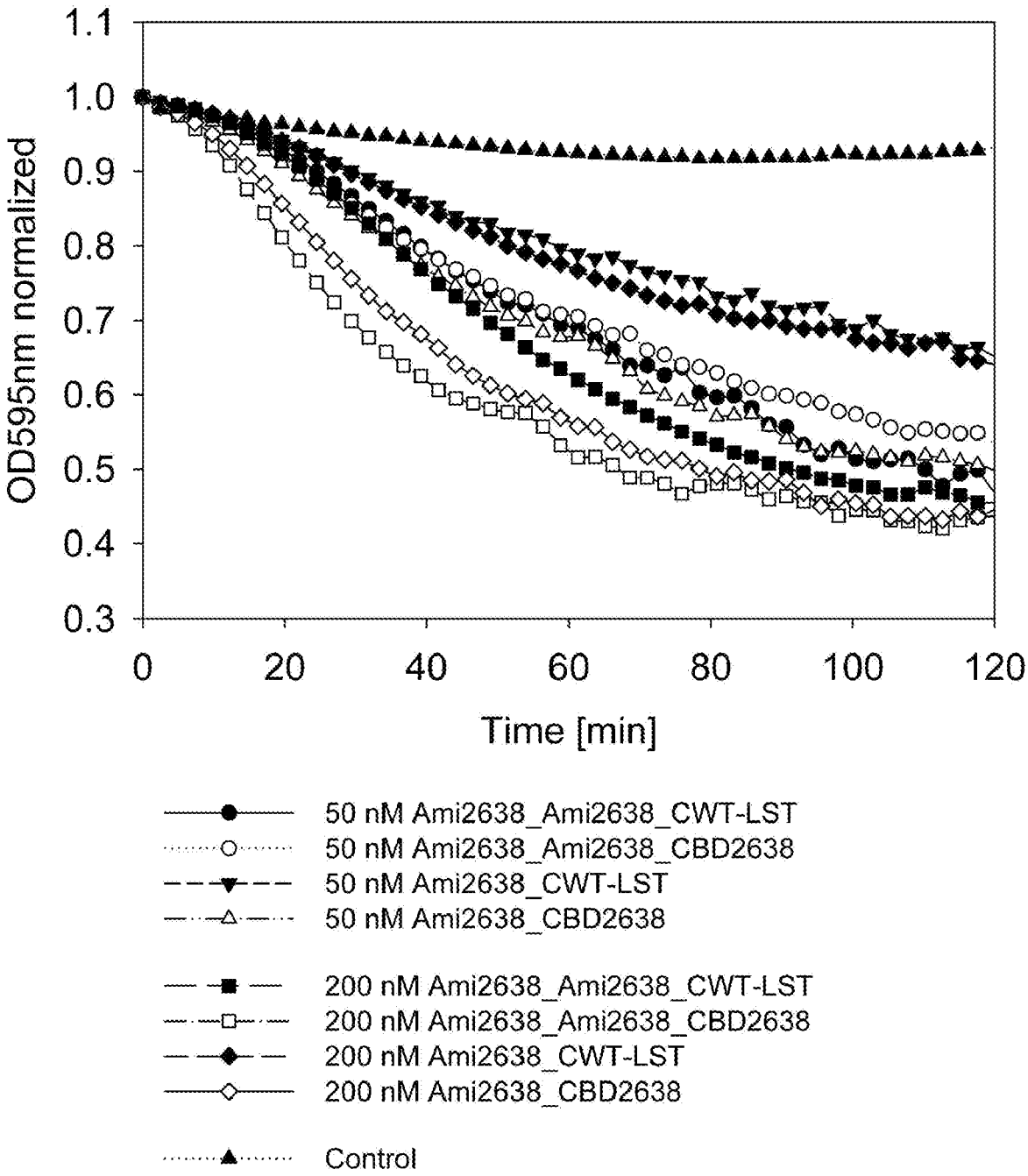

OD595nm normalized
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Time [min]
50 nM M23-LST_M23-LST_CWT-LST
50 nM M23-LST_M23-LST_CBD2638
50 nM M23-LST_CBD2638
200 nM M23-LST_M23-LST_CWT-LST
200 nM M23-LST_M23-LST_CBD2638
200 nM M23-LST_CBD2638
Time [min] vs Control

*Fig 7*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0.9
0.8
0.7
0.6
0.5
0.4
0.3
OD595nm normalized
0
20
40
60
80
100
120
Time [min]
50 nM Ami2638_Ami2638_CWT-LST
50 nM Ami2638_Ami2638_CBD2638
50 nM Ami2638_CWT-LST
50 nM Ami2638_CBD2638
200 nM Ami2638_Ami2638_CWT-LST
200 nM Ami2638_Ami2638_CBD2638
200 nM Ami2638_CWT-LST
200 nM Ami2638_CBD2638
Control

*Fig 8*

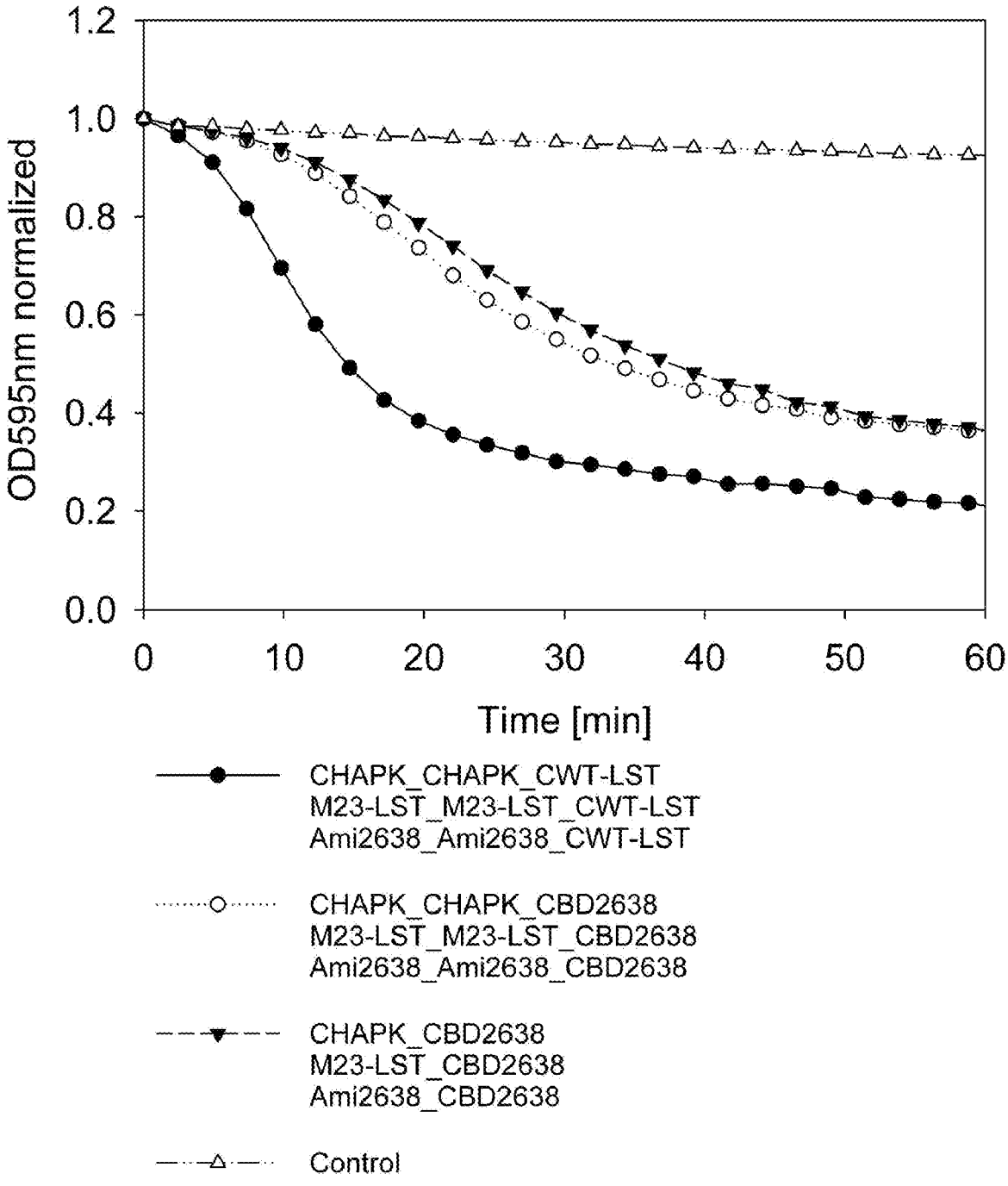

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD595nm normalized
0
10
20
30
40
50
60
Time [min]
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST
Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CBD2638
M23-LST_M23-LST_CBD2638
Ami2638_Ami2638_CBD2638
CHAPK_CBD2638
M23-LST_CBD2638
Ami2638_CBD2638
Control

*Fig 9*

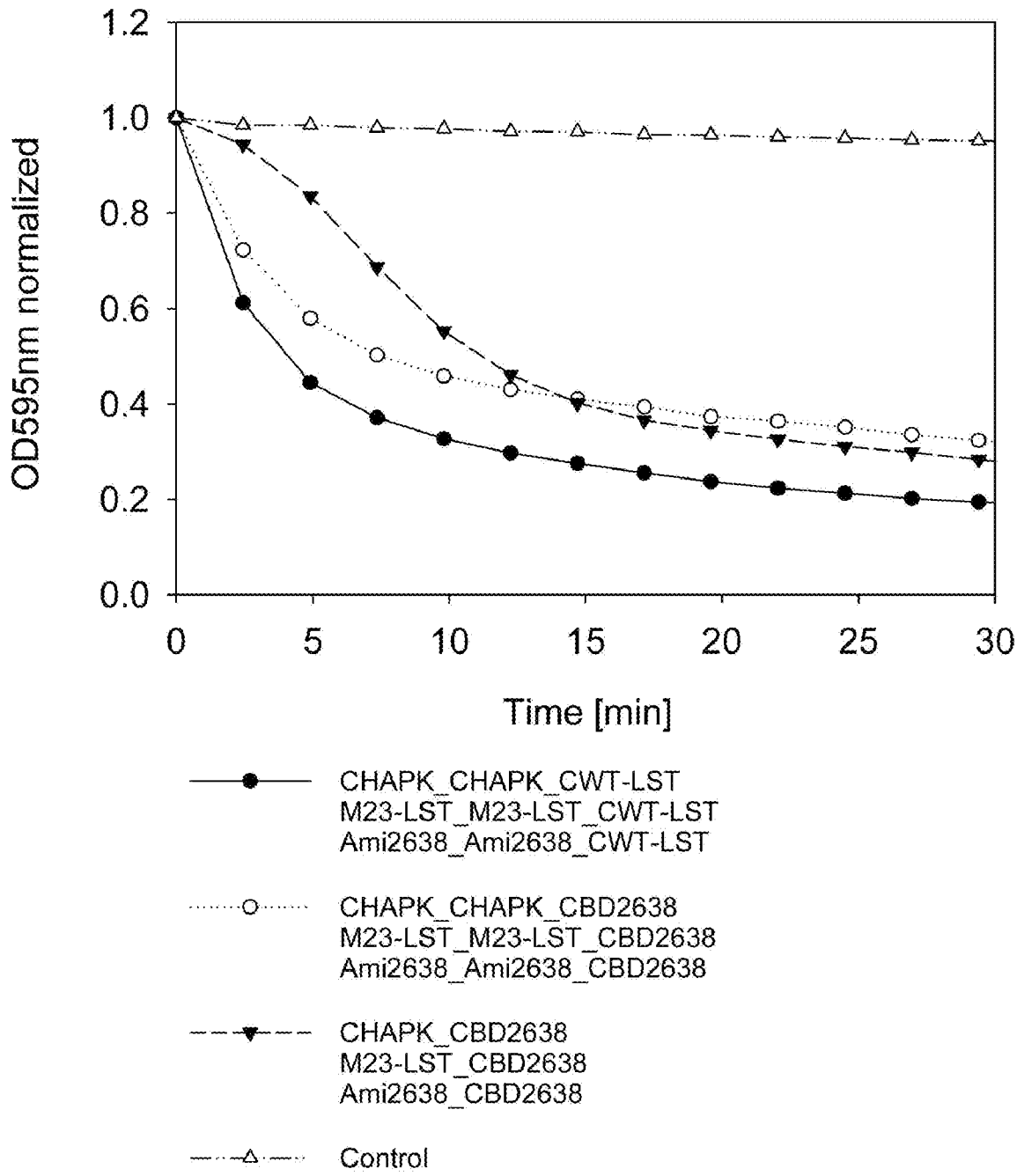

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD595nm normalized
0
5
10
15
20
25
30
Time [min]
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST
Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CBD2638
M23-LST_M23-LST_CBD2638
Ami2638_Ami2638_CBD2638
CHAPK_CBD2638
M23-LST_CBD2638
Ami2638_CBD2638
Control

*Fig 10*

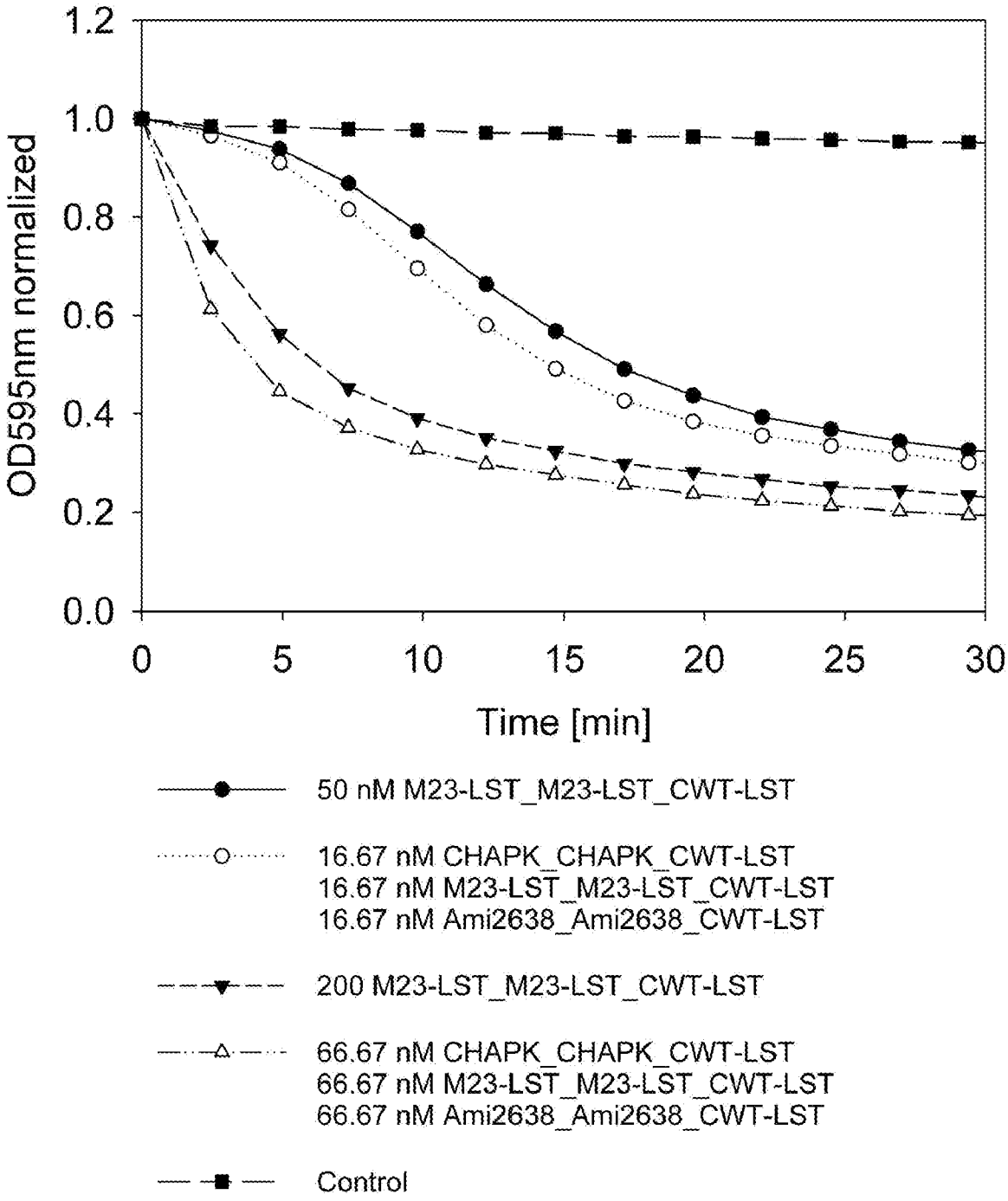

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD595nm normalized
0
5
10
15
20
25
30
Time [min]
50 nM M23-LST_M23-LST_CWT-LST
16.67 nM CHAPK_CHAPK_CWT-LST
16.67 nM M23-LST_M23-LST_CWT-LST
16.67 nM Ami2638_Ami2638_CWT-LST
200 M23-LST_M23-LST_CWT-LST
66.67 nM CHAPK_CHAPK_CWT-LST
66.67 nM M23-LST_M23-LST_CWT-LST
66.67 nM Ami2638_Ami2638_CWT-LST
Control

Fig. 11

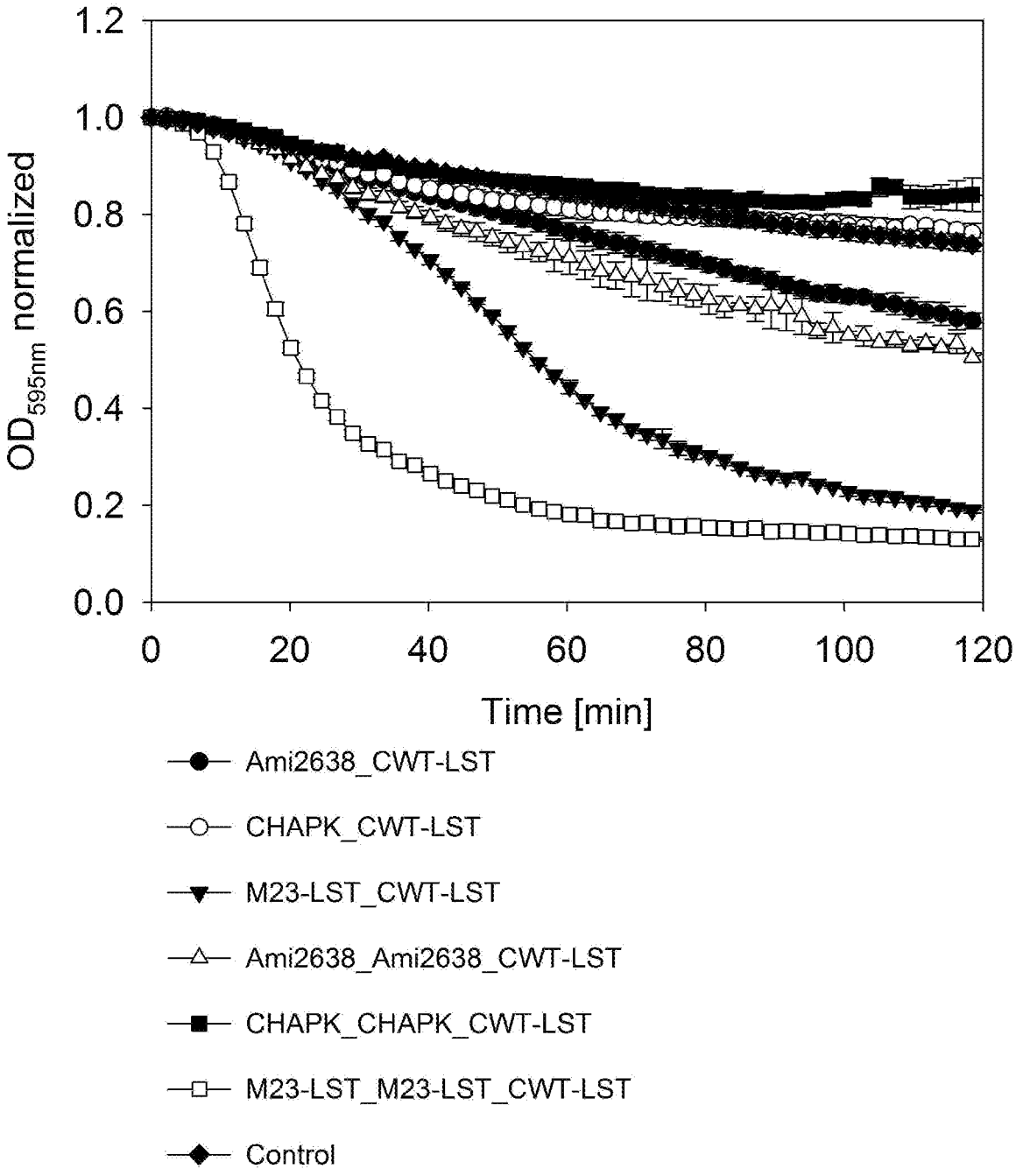
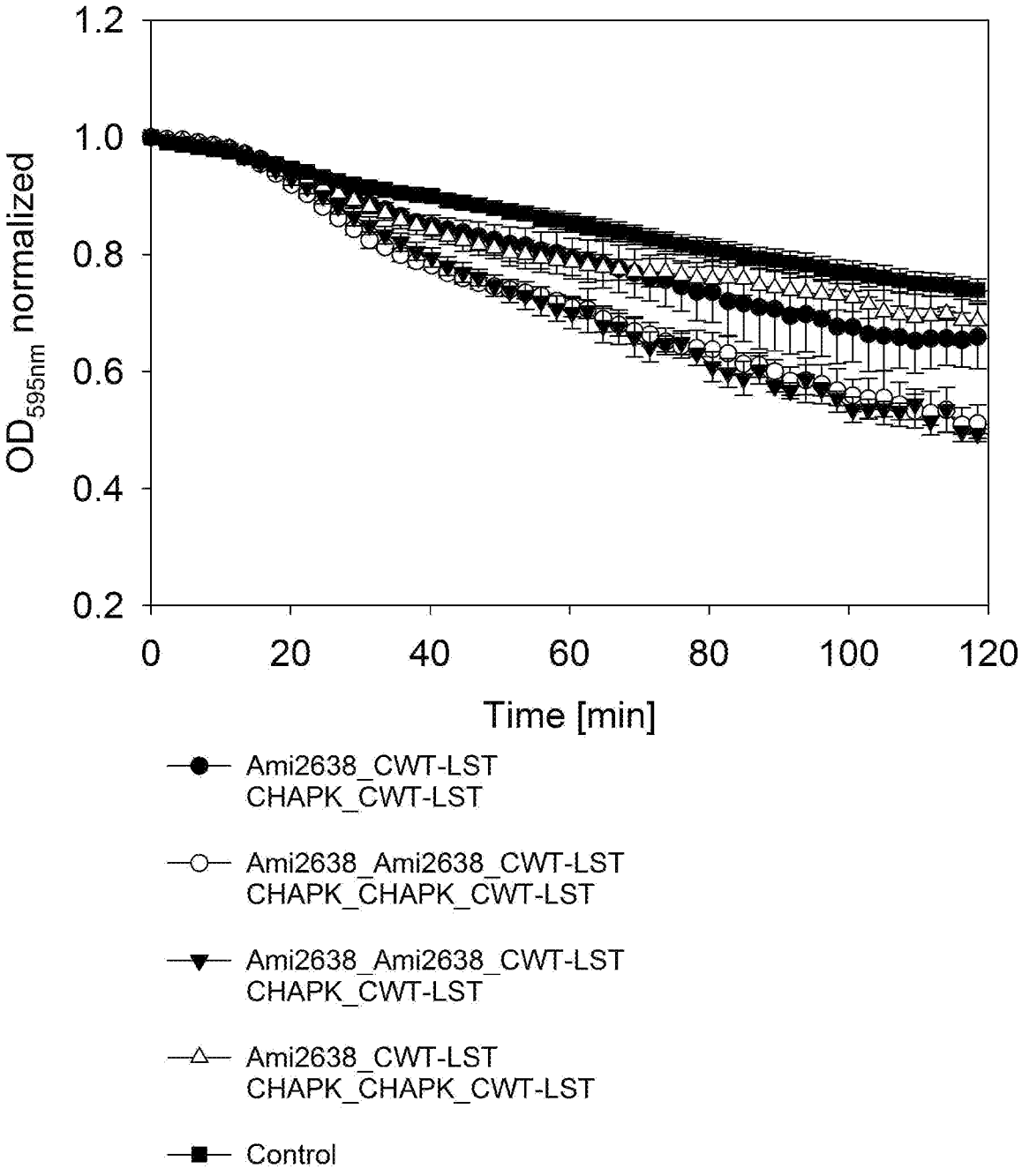

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD595nm normalized
0
20
40
60
80
100
120
Time [min]
Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_CWT-LST
Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST
Control

*Fig. 12*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0.8
0.6
0.4
0.2
OD595nm normalized
0
20
40
60
80
100
120
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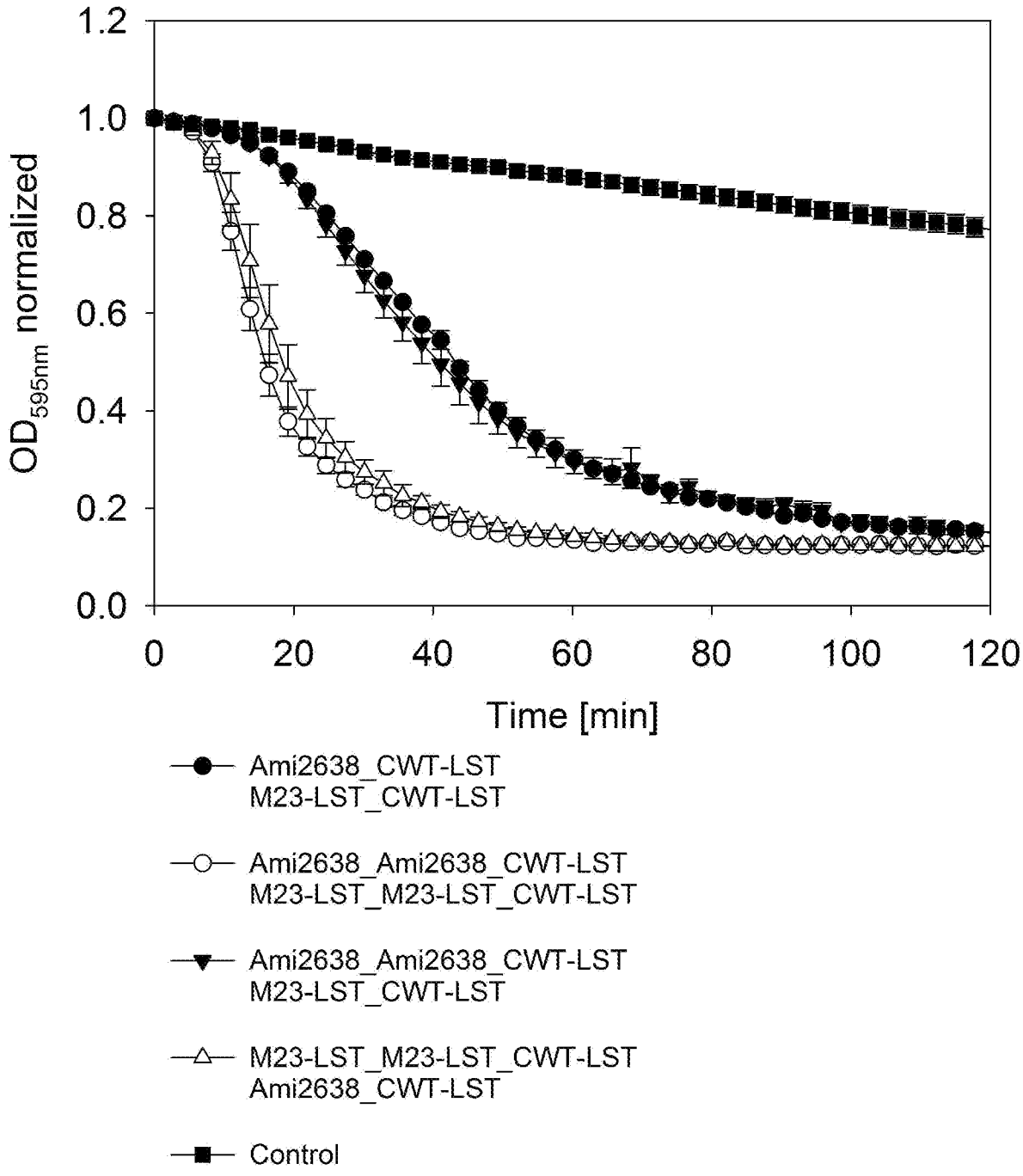
CHAPK_CWT-LST
Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
Ami2638_Ami2638_CWT-LST
CHAPK_CWT-LST
Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
Control

Fig. 13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0.8
0.6
0.4
0.2
0.0
OD595nm normalized
0
20
40
60
80
100
120
Time [min]
Ami2638_CWT-LST
M23-LST_CWT-LST
Ami2638_Ami2638_CWT-LST
M23-LST_M23-LST_CWT-LST
Ami2638_Ami2638_CWT-LST
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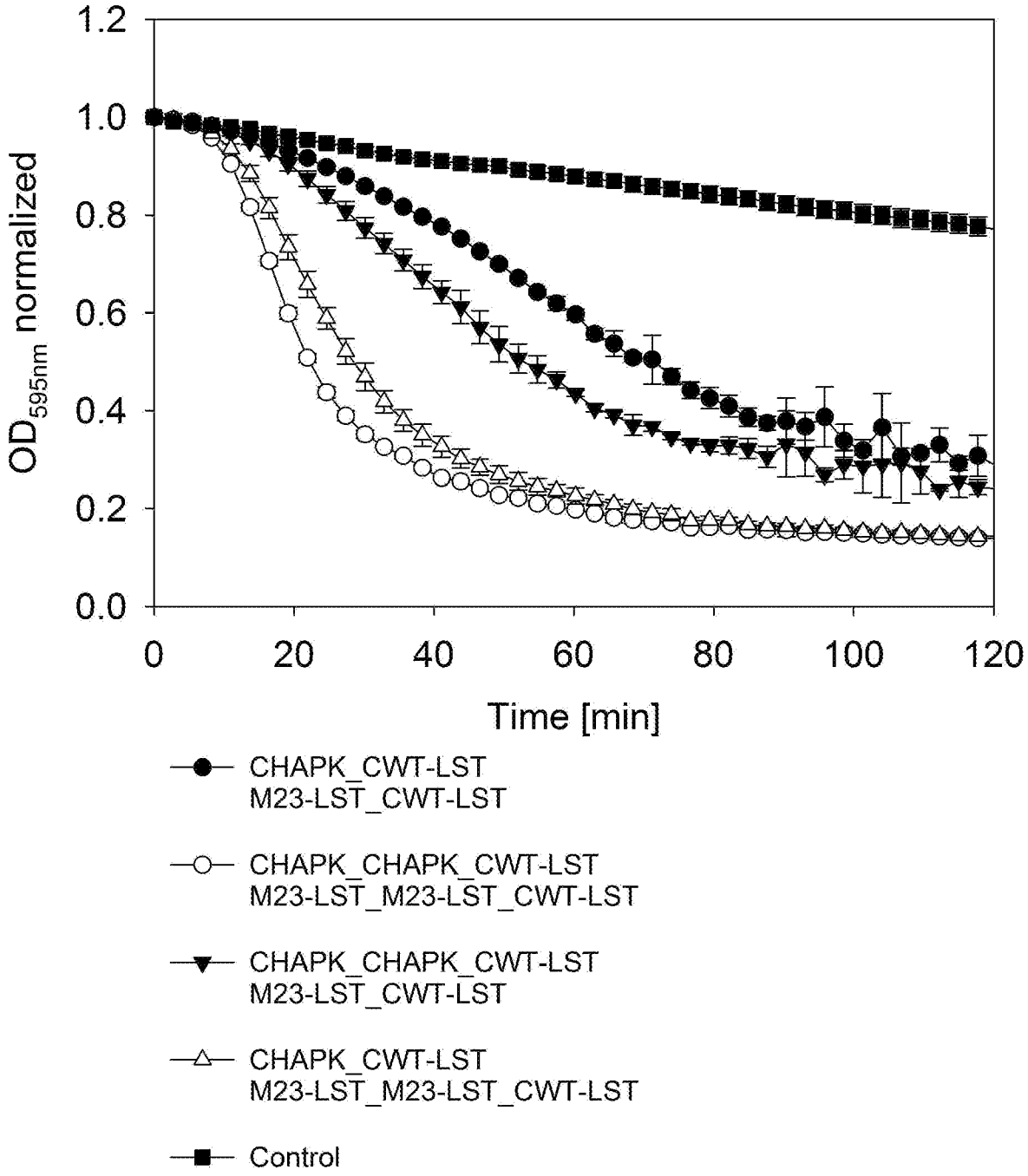
Ami2638_CWT-LST
Control

*Fig. 14*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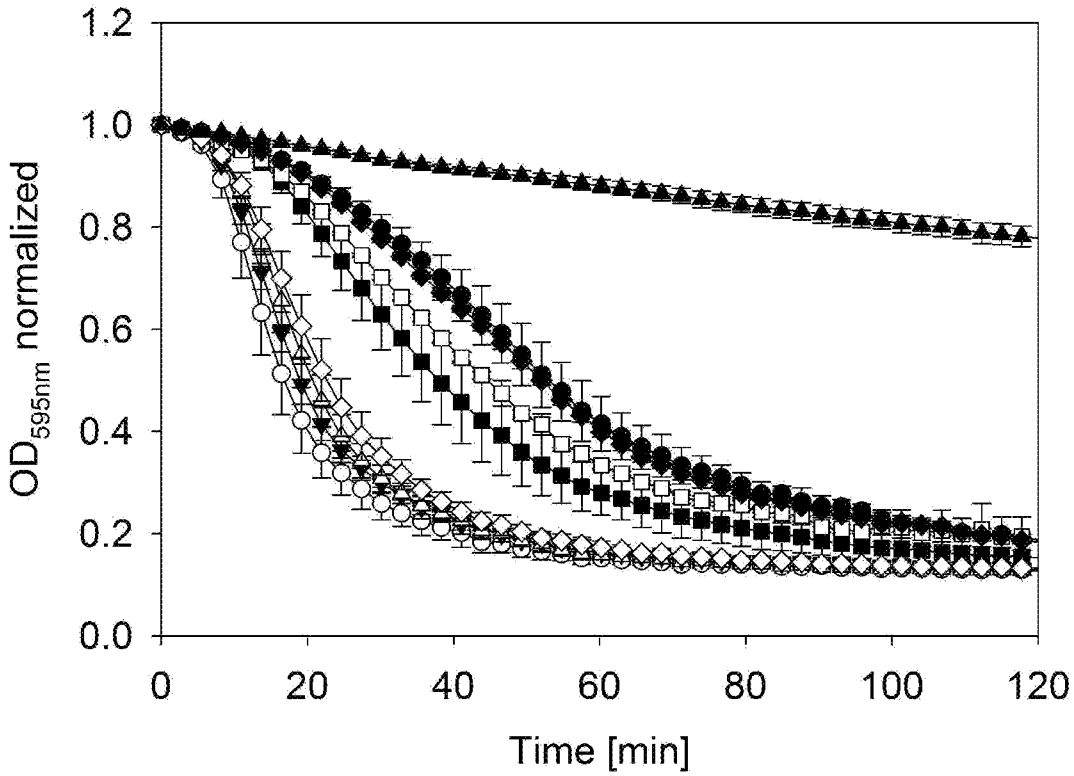
0.6
0.4
0.2
0.0
OD595nm normalized
0
20
40
60
80
100
120
Time [min]
CHAPK_CWT-LST
M23-LST_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_CWT-LST
CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST
Control

Fig. 15




Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_CWT-LST

Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST

Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST

Ami2638_Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST

Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_CWT-LST

Ami2638_Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_CWT-LST

Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_CWT-LST

Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST

Conrtol

Fig. 16

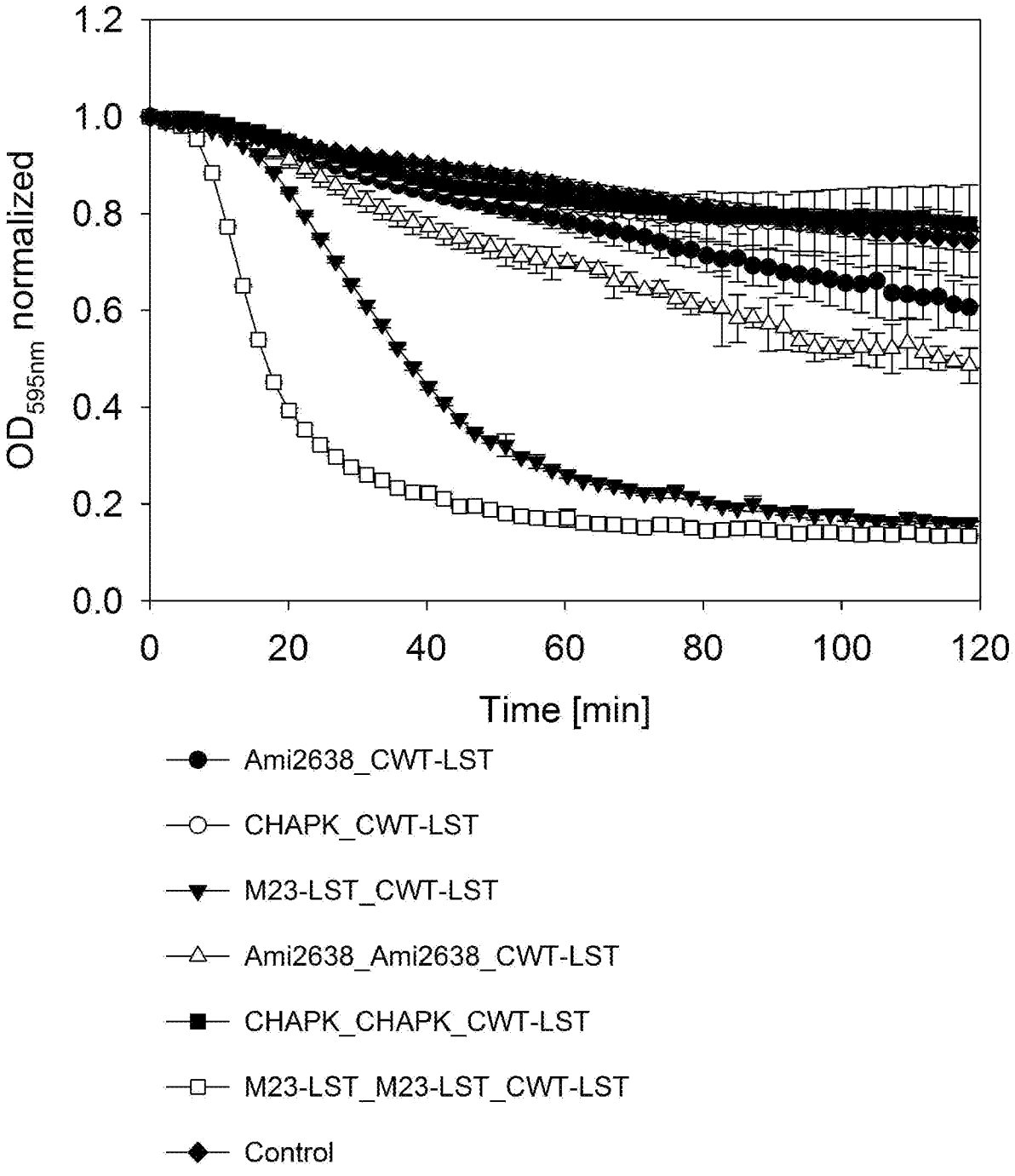

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD595nm normalized
0
20
40
60
80
100
120
Time [min]
Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_CWT-LST
Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST
Control

Fig. 17

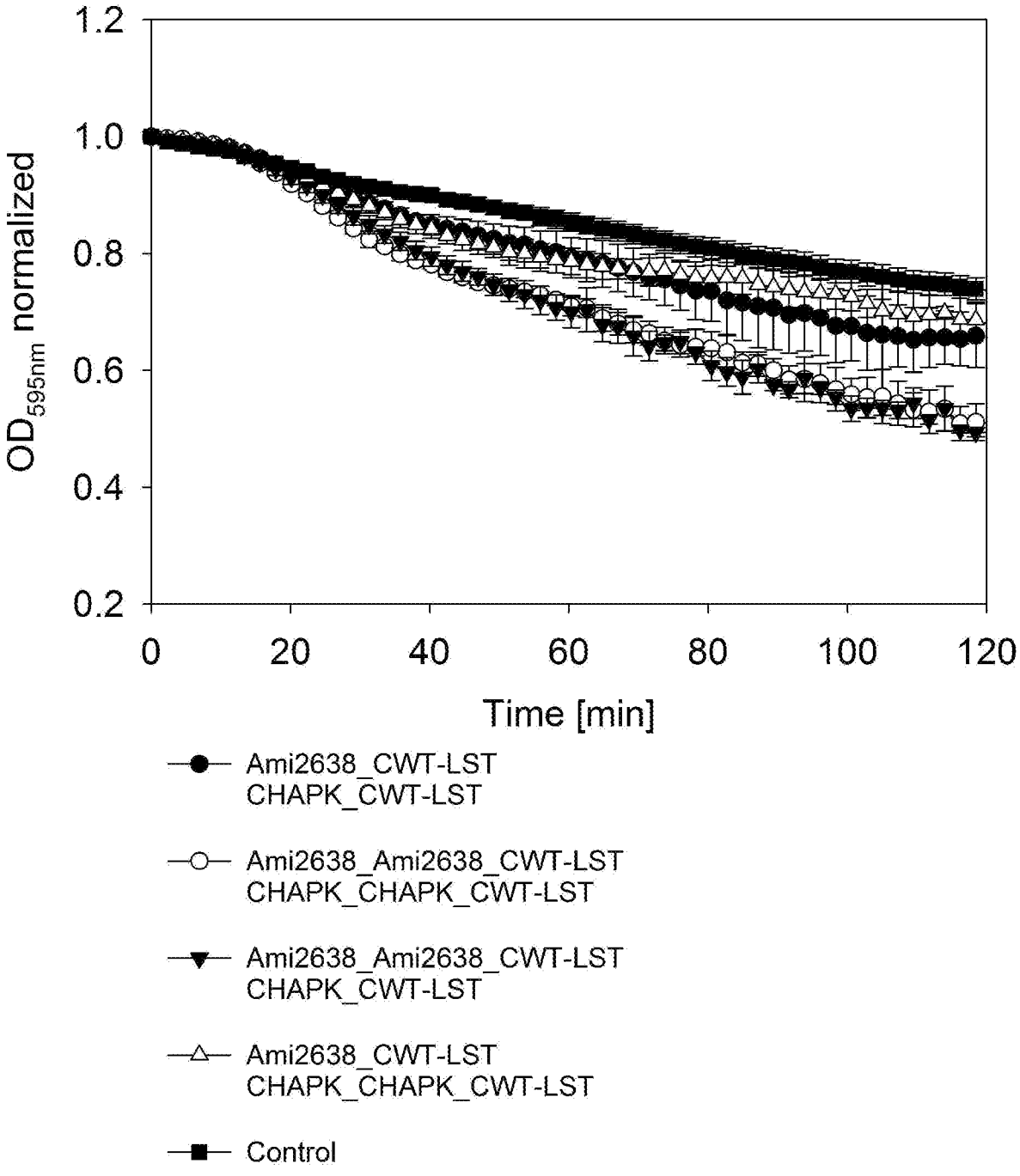

1.2
1.0
0.8
0.6
0.4
0.2
OD595nm normalized
0
20
40
60
80
100
120
Time [min]
Ami2638_CWT-LST
CHAPK_CWT-LST
Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
Ami2638_Ami2638_CWT-LST
CHAPK_CWT-LST
Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
Control

Fig. 18

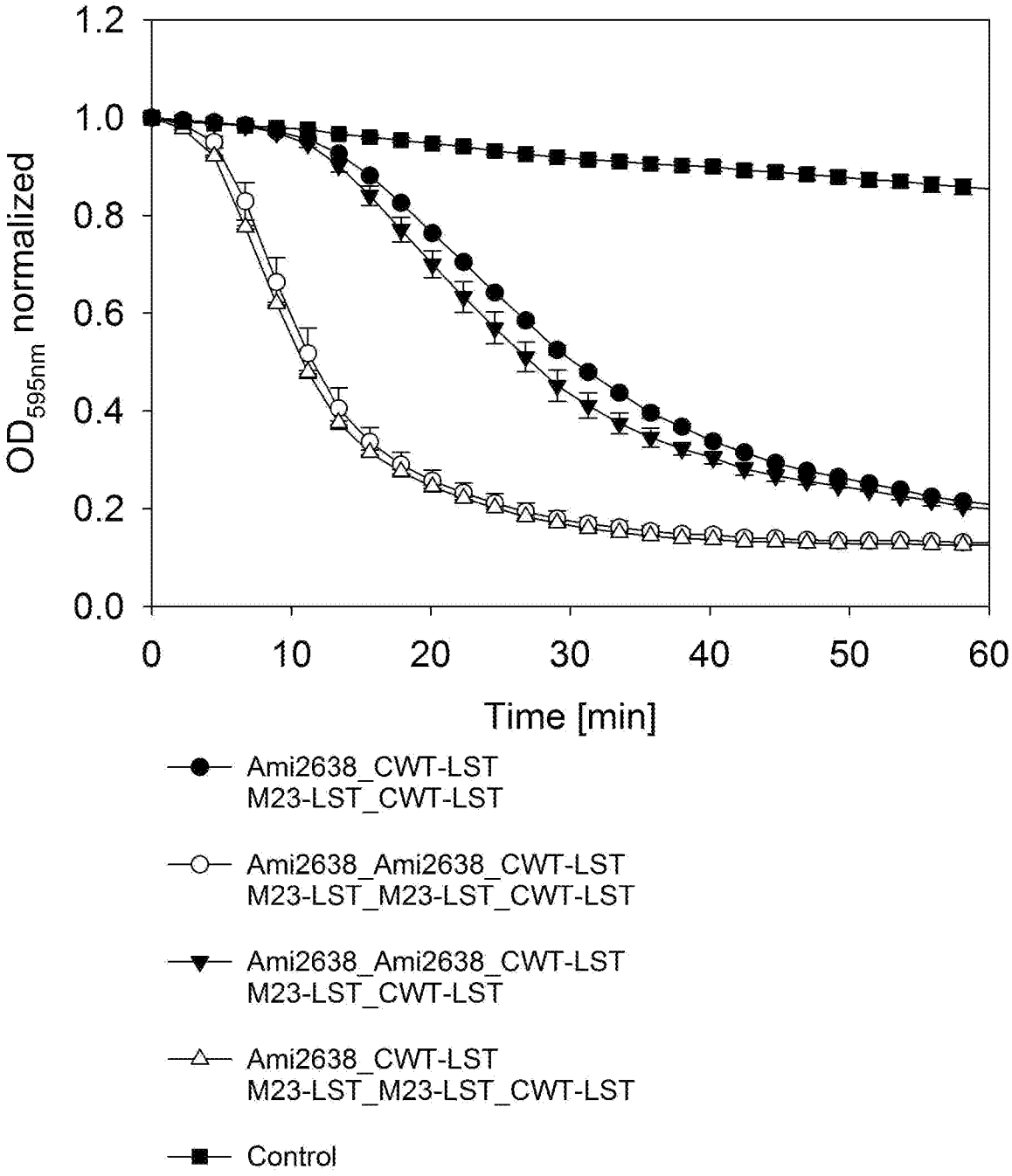
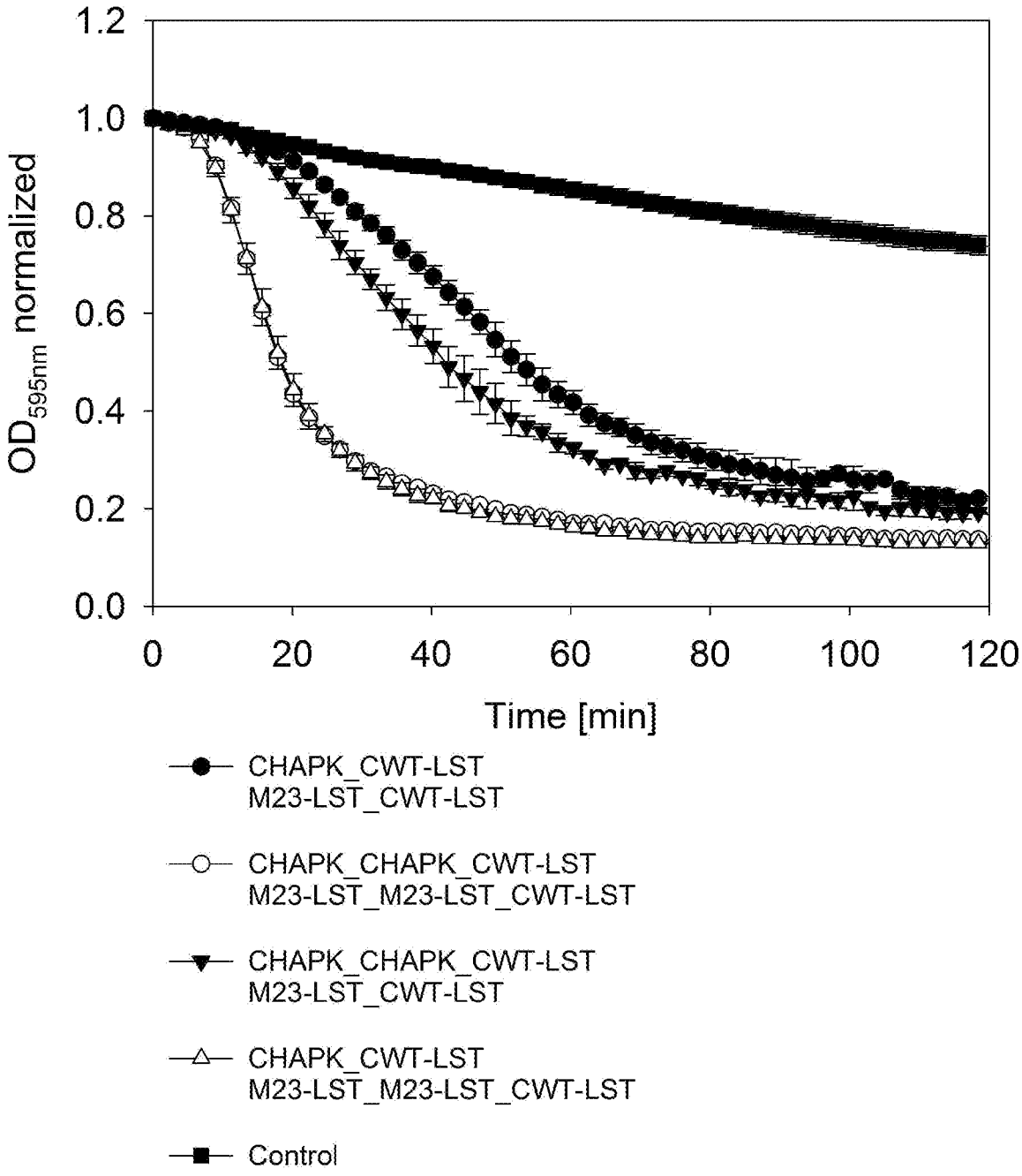
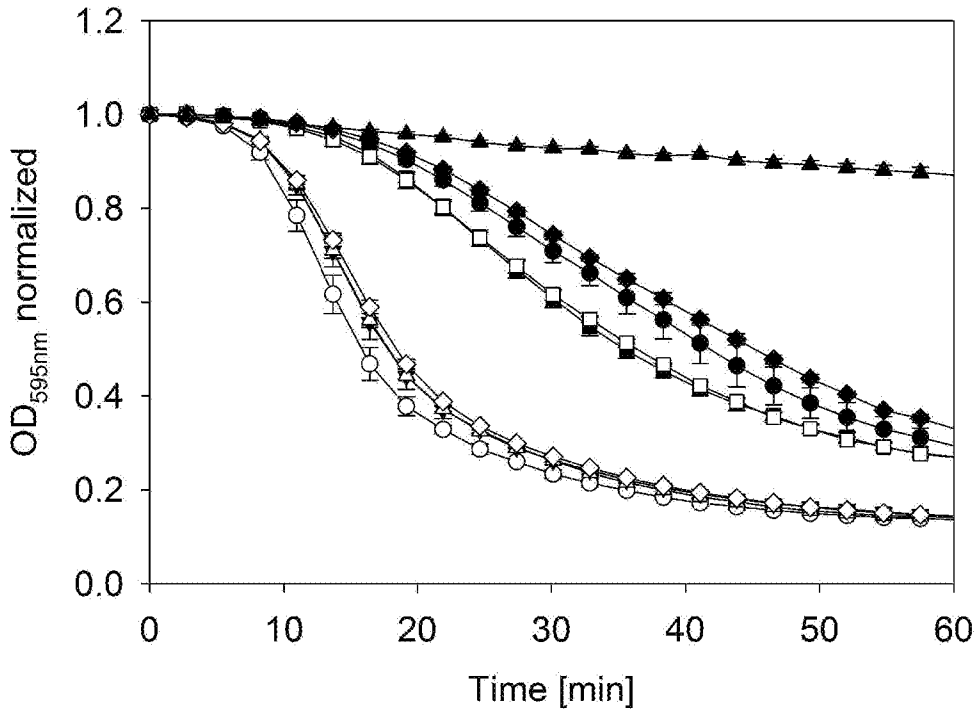

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD595nm normalized
0
10
20
30
40
50
60
Time [min]
Ami2638_CWT-LST
M23-LST_CWT-LST
Ami2638_Ami2638_CWT-LST
M23-LST_M23-LST_CWT-LST
Ami2638_Ami2638_CWT-LST
M23-LST_CWT-LST
Ami2638_CWT-LST
M23-LST_M23-LST_CWT-LST
Control

Fig. 19


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD595nm normalized
0
20
40
60
80
100
120
Time [min]
CHAPK_CWT-LST
M23-LST_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_CWT-LST
CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST
Control

# Fig. 20






Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_CWT-LST

Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST

Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST

Ami2638_Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST

Ami2638_Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_CWT-LST

Ami2638_Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_CWT-LST

Ami2638_CWT-LST
CHAPK_CHAPK_CWT-LST
M23-LST_CWT-LST

Ami2638_CWT-LST
CHAPK_CWT-LST
M23-LST_M23-LST_CWT-LST

Control

# INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2013/050344

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/50 C12N9/80 A61K38/51 A61K38/52 A61K38/16
A61K38/54 A23B4/22
ADD.
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)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | SCHMELCHER MATHIAS ET AL: "Chimeric Phage Lysins Act Synergistically with Lysostaphin To Kill Mastitis-Causing Staphylococcus aureus in Murine Mammary Glands", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 78, no. 7, April 2012 (2012-04), pages 2297-2305, XP009162654, ISSN: 0099-2240 the whole document<br>-----<br>-/-- | 1-15 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 8 July 2013 | 15/07/2013 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Bilang, Jürg |

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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Information on patent family members

International application No
PCT/NL2013/050344

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | CA 2736062 A1 | 28-01-2010 |
| | | EP 2318531 A2 | 11-05-2011 |
| | | US 2010158886 A1 | 24-06-2010 |
| | | WO 2010011960 A2 | 28-01-2010 |
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