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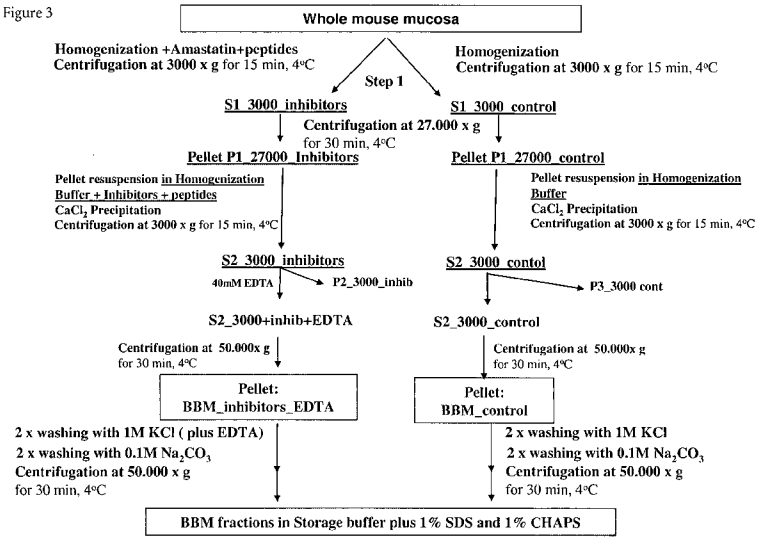
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(54) **Title:** METHOD FOR PURIFYING BRUSH BORDER MEMBRANE (BBM) PROTEINS

Figure 3



(57) **Abstract:** The present invention relates to a method for purifying brush border membranes comprising the following steps: a) providing tissue comprising brush border membranes; b) adding an inhibitor of microvillar membrane peptidases to the tissue; c) homogenizing the tissue; d) subjecting the homogenate to a low speed centrifugation resulting in a pellet and a supernatant; e) subjecting the supernatant of step d) to a high speed centrifugation resulting in a pellet and a supernatant; f) resuspending the pellet of step e); g) subjecting the resuspended pellet of step f) to a $CaCl_2$ or $MgCl_2$ precipitation resulting in a suspension; h) subjecting the suspension of g) to a low speed centrifugation resulting in a pellet and a supernatant; i) subjecting then the supernatant of step h) to a high speed centrifugation resulting in a pellet and supernatant; and k) washing the pellet of step i).

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METHOD FOR PURIFYING BRUSH BORDER MEMBRANE (BBM) PROTEINS

A brush border is the name for the microvilli-covered surface of pseudostratified columnar epithelium found in multiple locations of the body. They are found in two main locations: the small intestine tract and the kidney. The brush borders of the intestinal lining are the site of terminal carbohydrate digestions. The microvilli which constitute the brush border have enzymes for this final part of digestion anchored into their apical plasma membrane as integral membrane proteins. These enzymes are found near to the transporters which will then allow absorption of the digested nutrients. In the kidney, the brush border is useful in distinguishing the proximal tubule (which possesses the brush border) from the distal tubule (which does not).

Many proteolytic enzymes are constitutionally located in the Brush Border Membrane and cleave protein for transport through the small intestine. Many of those peptidases, such as Aminopeptidase N, Aminopeptidase A, Neprylisin, Di-Peptidyl-Protease IV, and Trehalase, are not inhibited by the more common (Ser-, Cys-) proteinase inhibitors. These peptidases could be inhibited chemically but only in anhydrous conditions. Protein degradation, however, is a major problem for relative quantification: Even a western blot will provide erroneous answers if the target protein is degraded.

These problems may be avoided with the present invention. Therefore, the present invention provides a method for purifying brush border membranes and thereby enriching the brush border membrane proteins. The method comprises the following steps:

a) providing tissue which comprises brush border membranes;

b) adding an inhibitor of microvillar membrane peptidases to the tissue;

c) homogenizing the tissue;

d) subjecting the homogenate to a low speed centrifugation resulting in a pellet and a supernatant;

e) subjecting the supernatant of step d) to a high speed centrifugation resulting in a pellet and a supernatant;

f) resuspending the pellet of step e);

g) subjecting the resuspended pellet of step f) to a $CaCl_2$ or $MgCl_2$ precipitation resulting in a suspension;

h) subjecting the suspension of g) to a low speed centrifugation resulting in a pellet and a supernatant;
i) subjecting then the supernatant of step h) to a high speed centrifugation resulting in a pellet and supernatant; and
k) washing the pellet of step i).

Preferably, an inhibitor of microvillar membrane peptidases (MMPs) is additionally added in step f) before $CaCl_2$ or $MgCl_2$ precipitation. The MMPs inhibitor may be added for example to the buffer with which the pellet of step e) is resuspended in step f). Most preferably, the MMPs inhibitor is added to each processing step (steps b) to k)) of the method of the invention.

The preferred MMPs inhibitor is amastatin.

The preferred concentration of the MMPs inhibitor is higher than 0.1mM. More preferably, the concentration of the MMPs inhibitor is between 0.5mM to 1.1mM. Most preferably the concentration is 1mM. When the MMPs inhibitor is added at more than one step of the method (e.g. at step b) and step f); or at each one of step b) to step k)), the concentration of added MMPs inhibitor may be different for the individual steps. However, preferably, the MMPs inhibitor is added in the same concentration.

Peptidase substrates may also be added to various steps of the method of the invention. Such substrate may for example be Angiotensin 1-5, Ala-Pro-PNA or Met-Bradykinin, or a mixture thereof. Preferably, the substrates are added additionally to the inhibitor. Also preferably, a peptidase substrate is added in step b) and/or step f). The peptidase substrate may be added in step b) for example dissolved in a buffer to the tissue. In step f) the peptidase substrate may be added for example to the buffer with which the pellet of step e) is resuspended.

Furthermore, in preferred embodiment, a chelating agent is added to the supernatant after the low speed centrifugation of step h) (supernatant resulting from step h)). The preferred chelating agent is EDTA.

A tissue which comprises brush border membranes is e.g. the mucosa tissue. The term "mucosa tissue" as used herein refers to the mucous tissue or mucous membrane of the small intestine. The mucous tissue/membrane is the innermost layer of the gastrointestinal tract, surrounding the lumen, or space within the tube. It comprises the epithelium, the lamina propria and a muscle layer. The epithelium is folded and forms so called villi wherein each of the surfaces of the villi is also folded to form microvilli. The

epithelium of these microvilli consists essentially of brush border cells (see also Figure 1). Brush border membrane is the cell membrane of said epithelial cells.

The term "intestine" refers to the portion of the alimentary canal extending from the stomach to the anus and, in humans and other mammals, consists of two segments, the small intestine and the large intestine. The small intestine is the portion of the intestine closest to the stomach. The small intestine has three structures to handle absorption: villi, microvilli and the circular folds.

Mucosa tissue may provided by scrapping the inner lining of the small intestine. The tissue may be isolated from proximal, central or distal section of the small intestine whereby the proximal section is the part nearest to the stomach and the distal section farthest from the stomach. For example, the provided mucosa tissue may derive from a pool of proximal, central and distal section of the small intestine or it may derive from only one or two of those sections.

The small intestine may be the small intestine of any animal. Preferably, it is the small intestine of a rodent or a bovine. More preferably, it is the intestine of a mouse or a cow.

The term "peptidase" refers to an enzyme which hydrolyzes peptide bonds between amino acids. Such a peptidase catalyzes the splitting of proteins or peptide into smaller peptide fractions and amino acids.

Microvillar membrane peptidases (MMPs) are peptidases which located within the cells of the brush border membrane. These peptidases are set free when the cell membrane is damaged, e.g. when the mucosa tissue is homogenized. Examples for such MMPs are Aminopeptidase N, Aminopeptidase A, Neprylisin, Di-Peptidyl-Protease IV, and Trehalase.

The term "inhibitor" refers to a molecule which represses or prevents another molecule from engaging in a reaction. The term "inhibitor of MMPs" refers to a peptidase inhibitor which represses or prevents to proteolysis of proteins by MMPs wherein said inhibitor is not a Serine- or a Cysteine-proteinase inhibitor.

A Serine-proteinase is a proteinase that is characterised by the presence of a serine residue in the active site of the enzyme. A Cysteine- proteinase is a proteinase that is characterised by the presence of a cysteine residue in the active site of the enzyme.

The term "homogenization" refers to a process that involves breaking apart cells and thereby releasing organelles and cytoplasm.

Methods for homogenization of membranes are well known to the skilled in the art. Such methods comprise for example osmotic alteration of the media in which the cells are or they comprise the use of physical force to disrupt cell structure. The physical means encompass use of mortars and pestles, blenders, compression and/or expansion, or ultrasonification (see e.g. Kessler M., Acuto O., Storelli C., Murer H., Müller M., and Semenza, G. *Biochimica and Biophysica Acta,* 506 (1978) 136-154.). Usually, tissue is homogenized in the presence of a buffer.

The term "low speed centrifugation" refers to a centrifugation at a speed of 1000 x g to 5000 x g Preferred speed is 2000x g to 4000 x g. An even more preferred speed is 3000 x g.

The term "high speed centrifugation" refers to a centrifugation at a speed of 20'000 x g to 70'000xg. Preferred speed is 30'000xg to 60000xg. An even more preferred speed is 50'000xg.

The term "buffer" refers to a solution containing either a weak acid and its salt or a weak base and its salt, which is resistant to changes in pH. Weak acids are not completely ionized, and a solution of a weak acid has a relatively low concentration of hydrogen ions. A weak base is a base that reacts only partially in an aqueous solution to produce an OH^- ion and the conjugate acid. A buffer is for a example a Tris buffer which comprises trishydroxymethylaminomethane (Tris) a buffer compound.

Preferably, pellet of step i) is washed with a high salt solution and high pH solution, both without calcium. Preferably, the salt solution comprises a chelating agent. The preferred chelating agent is EDTA. High salt solution means a salt concentration equal or higher than 1M for salts with monovalent cations and equal or higher than 0.5M for divalent cations. Preferably, the high salt solution is a KCl solution. A high pH solution means a solution with a pH value between 10 and 12. Preferably the pH value is 11. The preferred high pH solution is a Na_2CO_3 solution. Preferably, the pellet of step i) is washed first with a high salt solution and then with a high pH solution. In a preferred embodiment, the pellet is washed 1 to 3 times with each of the solutions, more preferably the pellet is washed twice.

The washed pellets of step k) comprise purified BBM membranes. These membranes, usually available as BBM vesicles, comprise enriched BBM proteins. The protein may be isolated from membranes with methods used for isolating other membrane proteins. Such methods are well known to the skilled in the art. A preferred method uses detergents such SDS or CHAPS.

The BBM proteins may be identified and relatively quantified by methods well known to the skilled in the art. For example, the BBM proteins are separated by 1D SDS PAGE and then digested with a site-specific endopeptidase, preferably Trypsin. The digested peptides are extracted and measured by LC-MS/MS.

The method of invention allows to purify the brush border membrane and thereby to enrich the brush border membrane proteins with less loss of proteins to degradation by peptidases. The method is a valuable tool for identifying brush border membrane proteins which are present in a low concentration and for the relative quantification of the membrane proteins.

The present invention further provides purified brush border membrane obtainable by the methods as described above. In addition, the present invention provides enriched brush border membrane proteins obtainable by the above described methods.

Furthermore, the present invention provides the use of amastatin for purifying brush border membrane.

Having now generally described this invention, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures.

Figures:

Figure 1 shows a schematic representation of a mucosa membrane. The mucous membrane comprises an epithelium. Said epithelium is folded and forms so called villi wherein each of the surfaces of the villi is also folded to form microvilli. The epithelium of these microvilli consists essentially of brush border cells.

Figure 2 shows a flow chart of the purifying method. Tissue comprising mucosa tissue is provided (preferably "total mucosa" = pooled from proximal, central and distal sections of the small intestine). Peptidase inhibitor is added and the tissue homogenated (step 1). The homogenized tissue is centrifuged at low speed (step 2) and then the supernatant resulting from the low speed centrifugation is centrifuged a high speed (step 3). The pellet resulting from the high speed centrifugation is resuspended, said resuspended pellet is subjected to a $CaCl_2$ precipitation or $MgCl_2$ precipitation and then the resulting suspension of the precipitation is centrifuged at low speed (step 4). The supernatant resulting from the low speed centrifugation is centrifuged a high speed (step 5) and the resulting pellet is washed (step 6).

Figure 3 shows a flow chart of a preferred embodiment of the purifying method.

Figure 4 shows a 1D SDS PAGE of purified brush border membrane (BBM) proteins. Lane M = Marker, Lane I = BBM control (without MMPs inhibitor), Lane II = with MMPs Inhibitor and EDTA, Lane III = BBM control washed (without MMPs inhibitor with washing step), Lane IV = with MMPs inhibitor and EDTA and washing step.

Figure 5 shows monitoring protein degradation by western blot of Aminopeptidase N. The primary antibody was a polyclonal antibody against the whole protein. Figure 5A shows the Aminopetidase N signal at the starting homogenized sample. Figure 5 B and C show respectively the Aminopeptidase N (APN) signal in a dilution series of the control BBM fraction and the BBM fraction in the presence of MMPs inhibitors. The APN signal is indicated by an arrow.

Lane M = Marker, Lane 1 = homogenized control (without MMPs inhibitor), 1µg; Lane 2= homogenized sample treated with MMPs inhibitor and peptidase substrates, 1µg Lane 3-6= BBM control, Lane 3: 0.5µg, Lane 4 = 0.1µg; Lane 5:0.05µg; Lane 6: 0.01µg; Lane 7-11: BBM with MMPs inhibitor and substrates, Lane 7: 5µg, Lane 8 = 1µg; Lane 9: 0.5µg; Lane 10: 0.1µg; Lane 11: 0.05µg

Examples

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated.

Example 1: Isolation of membrane vesicles.

Brush Border Membrane (BBM) vesicles were isolated by calcium precipitation using a modified method of Kessler et al. (Kessler M., Acuto O., Storelli C., Murer H., Müller M., and Semenza, G. *Biochimica and Biophysica Acta,* 506 (1978) 136-154). Scrapped mucosa from the small intestine of wild type C57B/6J-Bom male mice (Taconic) was suspended in 50 mM Mannitol, 2mM Tris (pH: 7.1) in presence of protease inhibitor cocktail tablets (Roche product Cat. No. 11080733001), 0.1 mM PMSF, 1 mM Amastatin (Bachem) (Hooper N.M., Hesp R.J., and Tieku S., *Biochem. J., 298* (1994) 635-639) and the peptide substrates Angiotensin 1-5, Ala-Pro-PNA, and Met-Bradykinin, at 1 mM concentration each. The control conditions are without the addition of Amastatin, peptide substrates and EDTA (see Figure 3: flow diagram of BBM preparation). The suspension was homogenized four times 20 seconds using a Polytron (Kinemetica GmbH) at maximum speed with intervals of 40 seconds in ice. The homogenized solution was then centrifuged at 3,000 x g for 15 min at 4 °C and the cell debris was discarded. The supernatant was re-centrifuged at 27,000 x g for 30 min at 4 °C. The resulting pellet was then suspended in the above buffer and $CaCl_2$ was added to a concentration of 10 mM. After incubation on ice for 20 min, a low spin centrifugation was repeated as described above. EDTA was added to the supernatant to a concentration of 40 mM and was re-centrifuged at 27000 x g for 30 min at 4 °C. The pellet was successively washed with 1 M KCl in presence of 40 mM EDTA, with 1 M KCl, and twice with 0.1 M Na_2CO_3, with 30 min centrifugation at 27,000 g at 4 °C after each washing step. The purified BBM pellet was finally solubilized in storage buffer (100 mM Mannitol, 1 mM Heppes-Tris pH: 7.5, 1% SDS and 1% CHAPS) and stored at -20 °C.

A comparison of the degradation between control and the method with amastatin, peptide substrates are shown in Figure 4 (SDS PAGE) and 5 (Amonopeptidase N in dilution series).

Example 2: Gel electrophoresis and protein digestion

The control and the inhibitor/EDTA BBM preparations (before and after the washes) were analyzed by SDS-PAGE using a NuPAGE 10% Bis-Tris gel (Invitrogen).

The gels were stained by colloidal Coomassie Blue (Invitrogen). The gel lanes were systematically excised into sections (see: Figure 4). Proteins present in each gel piece were digested with trypsin and tryptic peptides were extracted with acetonitrile (ACN) and formic acid. The extracted peptide samples were dried out in a speed vac and were re-dissolved in aqueous solution that contains 2% ACN and 0.5% acetic acid, prior to analysis.

Example 3: Reverse phase liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS)

Each peptide mixture was separated by nanoLC (Diomex) and analyzed on-line by electrospray tandem mass spectrometry using an a LTQ-Orbitrap mass spectrometer (Thermo). The system consisted of a nano flow HPLC (Ultimate 3000, DIONEX). The column (75 μm x 100 mm) was packed in house with Reprosil 70 C18, 3 μm material.

Mobile phase A was 2% ACN and 0.5% acetic acid and mobile phase B was 80 % ACN and 0.5% acetic acid. Gradient elution was carried out from 5% B to 60% B in 75 min. Eluting peptides were detected and sequenced using an LTQ Orbitrap instrument (Thermo). The full scan survey was performed in the Orbitrap detector at 30,000 resolution while daughter spectra were acquired in the LTQ mass spectrometer.

Example 4: Data analysis

MS/MS spectra were processed through the SEQUEST software package (Thermo) against the a database comprising all mouse proteins using the following criteria: parent mass ± 10 ppm, daughter ions ± 0.5Da, tryptic cleavage specificity with one miss cleavage allowed. Peptides that had Xcorr of 1.5/2.0/2.5 for single/double/triple charged precursor and dCn>0.1 were accepted for further processing. A protein that was represented by at least two different peptides was considered confidently identified.

Example 5: Western Blot analysis

The separated proteins in 1D SDS-PAGE gel (see Example 2), were transferred to a PVDF membrane (Perkin-Ellmer) and probed with a goat polyclonal primary antibody against mouse aminopeptidase N (aa69-966), (R&D Systems). The secondary antibody

was a donkey anti-goat antibody, HRP-conjugated (Santa Cruz Biotechnology). Blots were developed with eclectrochemiluminescence (ECL) detection reagent according to manufacturer's instructions (Amersham Biosciences, France)

Results:

The modified $CaCl_2$ precipitation in combination with the high salt and high pH washes (Zhao Y., Zhang W., Kho Y., and Zhao Y., *Anal Chem.*, 76 (2004) 1817-1823) was very effective for the enrichment of very hydrophobic proteins, for example, some of the important transporters and receptors that have been described to play critical role in cholesterol absorption (table 1). The addition of Amastatin (a general aminopeptidase inhibitor) in combination with peptide substrates specifically chosen to compete for the highly abundant peptidases of the BBM membrane fraction (table 2), help to decrease protein degradation (see figure 5: western blot of Aminopeptidase N).

The partial inhibition of membrane peptidases such as Aminopeptidase N, DPP IV, Neprilysin, is also visible in the comparison of the number of identified different peptides for each protein and the number of times that this protein has been identified in each gel lane (see examples on table 3).

The protein identification of BBM vesicles has been the target of many recent publications (Babusiak M., Man P., Petrak J., and Vyoral D., *Proteomics,* 7 (2007) 121-129; Nguyen H.T.T., Amine A.B., Laffite D., Waheed A.A., Nicoletti C., Villard C., Létisse M., Deyris V., Roziére M., Tchiakpe L., Danielle C., and Comeau L., Hiol A., *Biochemical and Biophysical Research Communication,* 342 (2006) 236-244; Blonder J., Hale M.L., Lucas D.A., Schaefer C.F., Yu L., Conrads T.P, Issaq H.J, Stiles B.G, and Veenstra T.D., *Electrophoresis*, 25 (2004) 1307-1318) in the proteomic field, but so far there is none that shows identification of lower abundant proteins such as receptors and transporters that participate in cholesterol absorption. The additional steps and the washes in the membrane preparation were essential for the identification of low abundant proteins. The inhibition of the BBM peptidases is critical for further studies in cholesterol absorption, where different cholesterol inhibitors are used and the protein abundance and distribution needs to be defined and compared quantitatively.

Even the partial inhibition of these resistant peptidases can enhance the reliability of a western blot technique. As an example, if an antibody is raised against a specific epitope, the protein signal in a western blot can decrease or disappear and/or degradation

products may be detected. This might lead to a false interpretation of the results if protein degradation is not considered.

Table 1: purified BBM proteins according method of invention (possible candidates of cholesterol absorption). Mass = mass in kDa of proteins, Coverage = the amino acid sequence coverage of the protein that is covered by the identified peptide; PepCount = sum of the times a petide has been identified for a given protein; DifferentPeps = number of different peptides that have been identified in a given protein; Occurrence = number of gel bands that a given protein has been identified. The rank order is representative of the level of abundance. Rank 1 is the most abundant protein with the highest peptide count and the most different peptides.

protein set	Protein Header	Mass	Coverage	Pep Count	Different Peps	Occurrence	Rank
11,170	mousegp:MCHR3-063958|--|SWTR_MUS:MOT1_MOUSE: Monocarboxylate transporter 1 (MCT 1) (Solute carrier family 16 member 1).	53.2	10.50%	12	4	4	200
2,186	mousegp:MCHR19-060871|--|SWTR_MUS:MRP2_MOUSE: Canalicular multispecific organic anion transporter 1 (ATP-binding cassette sub-family C member 2).	173.5	16.10%	51	19	4	72
5,181	mousegp:MCHR3-064878|--|SWTR_MUS:MTP_MOUSE: Microsomal triglyceride transfer protein large subunit precursor.	99	5.60%	4	4	1	417
6,187	mousegp:MCHR17-058540|--|SWTR_MUS:ABCG8_MOUSE: ATP-binding cassette sub-family G member 8 (Sterolin-2).	75.9	16.50%	26	9	5	110
6,187	mousegp:MCHR17-059274|--|SWTR_MUS:ABCG5_MOUSE: ATP-binding cassette sub-family G member 5 (Sterolin-1).	73.2	16.40%	15	10	1	166
3,171	mousegp:MCHR11-164370|--|SWTR_MUS:NPCL1_MOUSE: Niemann-Pick C1-like protein 1	147	23.60%	133	31	8	30
10,172	mousegp:MCHR8-073365|--|SWTR_MUS:NTCP2_MOUSE: Ileal sodium/bile acid cotransporter (Ileal Na(+)/bile acid cotransporter) (Na(+)-dependent ileal bile acid transporter) (Ileal sodium-dependent bile acid transporter) (ISBT) (IBAT)	38.1	9.20%	11	4	2	220
13,190	mousegp:MCHR2-063036|--|SWTR_MUS:ABCBB_MOUSE: Bile salt export pump (ATP-binding cassette sub-family B member 11) (Sister of P-glycoprotein).	146.9	1.90%	5	2	1	367
12,189	mousegp:MCHR5-166843|--|SWTR_MUS:MDR1_MOUSE: Multidrug resistance protein 1 (EC 3.6.3.44) (ATP-binding cassette sub-family B member 1) (P-glycoprotein 1) (CD243 antigen).	140.9	3.20%	3	3	1	472

protein set	Protein Header	Mass	Coverage	Pep Count	Different Peps	Occurrence	Rank
15,185	mousegp:MCHR9-086280|--|SWTR_MUS:APOA1_MOUSE: (Apo-AI) (ApoA-I).	30.6	8%	2	2	1	601
13,196	mousegp:MCHR7-071588|--|SWTR_MUS:APOE_MOUSE: Apolipoprotein E precursor (Apo-E).	35.8	13.20%	3	3	1	479
11,270	mousegp:MCHR9-074364|--|SWTR_MUS:APOA4_MOUSE: (Apo-AIV) (ApoA-IV).	45	8.10%	6	3	2	416
12,189	mousegp:MCHR7-063783|--|SWTR_MUS:LEG4_MOUSE: Galectin-4	36.3	34.70%	28	10	4	104
11,170	mousegp:MCHR14-080224|--|SWTR_MUS:ANXA7_MOUSE:	49.9	16.20%	14	6	2	170
7	mousegp:MCHR2-061149|--|SWTR_MUS:S27A4_MOUSE: Long-chain fatty acid transport protein 4 (EC 6.2.1.-) (Fatty acid transport protein 4) (FATP-4)	72.3	2.60%	2	2	1	558
6,187	**mousegp:MCHR5-084457|--|SWTR_MUS:SCRB1_MOUSE: Scavenger receptor class B member 1 (SRB1) (SR-BI).**	**56.7**	**2.40%**	**1**	1	**1**	**1016**

Table 2: Most abundant proteins in the BBM fraction. Peptidases are highlighted in grey. Mass = mass in kDa of proteins, Coverage = the amino acid sequence coverage of the protein that is covered by the identified peptide; PepCount = sum of the times a peptide has been identified for a given protein; DifferentPeps = the number of different peptides that have been identified in a given protein; Occurrence = The number of gel bands that a given protein has been identified. The rank order is representative of the level of abundance. Rank 1 is the most abundant protein with the highest peptide count and the most different peptides.

protein set	Protein Header	Mass	Coverage	Pep Count	Max Different Peps	Occurrence	Rank
3,171	mousegp:MCHR3-248581|--|SWTR_RAT:SUIS_RAT: Sucrase-isomaltase, intestinal	206.2	43.50%	1903	69	16	1
1,202	mousegp:MCHR6-167409|--|SWTR_HUM:MGA_HUMAN: Maltase-glucoamylase, intestinal	198.5	39.70%	1382	67	15	2
3,171	mousegp:MCHR7-072136|--|SWTR_MUS:AMPN_MOUSE: Aminopeptidase N (EC 3.4.11.2)	109.4	58.30%	1119	58	16	3
10,172	mousegp:MCHR11-079188|--|SWTR_MUS:ACTG_MOUSE: Actin, cytoplasmic 2 (Gamma-actin). -	41.7	70.70%	902	26	15	4
5,179	mousegp:MCHR1-047171|--|SWTR_MUS:VILI_MOUSE: Villin-1. -	92.6	53.40%	834	43	15	5
7,194	mousegp:MCHR5-066994|--|SWTR_MUS:SC5A1_MOUSE: Sodium/glucose cotransporter 1 (Na(+)/glucose cotransporter 1)	73.3	31.10%	622	22	12	6
1,202	mousegp:MCHR3-228712|--|SWTR_MUS:AT1A1_MOUSE: Sodium/potassium-transporting ATPase alpha-1 chain precursor	112.9	41.60%	531	37	14	8
4,174	mousegp:MCHR10-049404|--|SWTR_HUM:MYO1A_HUMAN: Myosin Ia (Brush border myosin I)	118.6	59.90%	456	70	13	9
3,171	mousegp:MCHR3-244828|--|SWTR_MUS:AMPE_MOUSE: Glutamyl aminopeptidase (EC 3.4.11.7) (EAP)	107.9	54.70%	427	59	15	10
10,172	mousegp:MCHR6-085038|--|SWTR_MUS:STYK1_MOUSE: Tyrosine protein-kinase STYK1 (EC 2.7.10.2) (Serine/threonine/tyrosine kinase 1) (Novel oncogene with kinase-domain) (mNOK). -	48.1	41.70%	384	14	4	11

protein set	Protein Header	Mass	Coverage	Pep Count	Max Different Peps	Occurrence	Rank
13,196	mousegp:MCHR16-057056|--|SWTR_MUS:MUC13_MOUSE: Mucin-13 precursor (Cell surface antigen 114/A10)	58.6	24.10%	365	11	15	12
5,179	mousegp:MCHR3-191469|--|SWTR_MUS:NEP_MOUSE: Neprilysin (EC 3.4.24.11) (Neutral endopeptidase) (NEP)	85.5	41.10%	355	30	13	13
2,186	mousegp:MCHR18-060419|--|SWTR_MUS:Q99MZ6_MOUSE: Myosin-VIIb. was used	240.1	34%	295	69	10	15
3,171	mousegp:MCHR5-243939|--|SWTR_MUS:MDR3_MOUSE: Multidrug resistance protein 3 (ATP-binding cassette sub-family B member 1A)	140.5	44.70%	287	50	12	16
6,187	mousegp:MCHR14-229808|--|SWTR_MUS:Q14BA3_MOUSE: Slc15a1 protein. -	78.5	22.80%	283	18	5	17
4,174	mousegp:MCHR18-229929|--|SWTR_MUS:MEP1B_MOUSE: Meprin A subunit beta precursor	79.4	36.60%	278	21	14	18
3,171	mousegp:MCHR1-163982|--|SWTR_RAT:LPH_RAT: Lactase-phlorizin hydrolase precursor (Lactase-glycosylceramidase)	217.6	26.90%	260	54	9	19
6,187	mousegp:MCHR1-227798|--|SWTR_MUS:PPBI_MOUSE: Intestinal alkaline phosphatase precursor (IAP).	60.2	44.70%	230	24	6	20
14,177	mousegp:MCHR10-270835|--|SWTR_MUS:Q0VG18_MOUSE: RIKEN cDNA 2210404O07	13.5	37.50%	227	6	4	21
4,174	mousegp:MCHRX-086939|--|SWTR_MUS:ACE2_MOUSE: Angiotensin-converting enzyme 2 precursor (EC 3.4.17.-) (ACE-related carboxypeptidase). -	92.3	53.30%	224	33	14	22
14,177	mousegp:MCHR11-051435|--|SWTR_MUS:Q6YI28_MOUSE: EP1 (Novel protein). -	23.6	26.40%	211	8	8	23
11,170	mousegp:MCHR10-137211|--|SWTR_MUS:GNAQ_MOUSE: Guanine nucleotide-binding protein G(q) subunit alpha (Guanine nucleotide-binding protein alpha-q). -	41.3	77.90%	204	36	1	24

protein set	Protein Header	Mass	Coverage	Pep Count	Max Different Peps	Occurrence	Rank
2,186	mousegp:MCHR13-263698|--|SWTR_MUS:Q3UN77_MOUSE: Adult male intestinal mucosa cDNA, RIKEN full-length enriched library, clone:G630055D06 product:similar to Protocadherin LKC. -	142.5	22.50%	176	26	15	25
11,170	mousegp:MCHR19-139763|--|SWTR_MUS:GNA11_MOUSE: Guanine nucleotide-binding protein alpha-11 subunit. -	42.1	69.10%	145	28	1	26
13,196	mousegp:MCHR6-069801|--|SWTR_MUS:ANXA4_MOUSE: Annexin A4 (Annexin IV). -	35.7	55%	140	21	7	27
3,171	mousegp:MCHR19-060184|--|SWTR_MUS:NALDL_MOUSE: N-acetylated-alpha-linked acidic dipeptidase-like protein (EC 3.4.17.21) (NAALADase L). -	80.4	33.30%	137	15	9	29
3,171	**f|--|SWTR_MUS:NPCL1_MOUSE: Niemann-Pick C1-like protein 1 precursor. -**	**147**	**23.6 %**	**133**	**31**	**8**	**30**
5,179	mousegp:MCHR17-165548|--|SWTR_MUS:Q91WV7_MOUSE: Solute carrier family 3, member 1	78.1	35.30%	114	23	9	31
4,174	mousegp:MCHR2-228597|--|SWTR_MUS:DPP4_MOUSE: Dipeptidyl peptidase 4 (EC 3.4.14.5) (Dipeptidyl peptidase IV) (DPP IV)	87.4	37%	112	30	10	32
3,171	mousegp:MCHR10-264555|--|SWTR_MUS:Q6DYE8_MOUSE: Ectonucleotide pyrophosphatase/phosphodiesterase 3.	98.6	43%	102	31	6	33
7,194	mousegp:MCHR10-048971|--|SWTR_MUS:GGT1_MOUSE: Gamma-glutamyltranspeptidase 1 precursor (EC 2.3.2.2)	61.5	27.30%	101	17	7	34
4,174	mousegp:MCHR11-079038|--|SWTR_MUS:MYO1D_MOUSE: Myosin Id.	116	47.20%	97	44	5	35
3,171	mousegp:MCHR19-228496|--|SWTR_MUS:ASAH2_MOUSE: Neutral ceramidase (EC 3.5.1.23) (NCDase) (N-CDase)	83.4	43%	95	20	8	36

protein set	Protein Header	Mass	Coverage	Pep Count	Max Different Peps	Occurrence	Rank
7,194	mousegp:MCHR8-073301|--|SWTR_MUS:DPEP1_MOUSE: Dipeptidase 1 precursor (EC 3.4.13.19) (Microsomal dipeptidase) (Renal dipeptidase) (Membrane-bound dipeptidase 1) (MBD-1)	45.7	50%	92	16	2	37
7,194	mousegp:MCHR15-165261|--|SWTR_MUS:Q3UV17_MOUSE: Adult female vagina cDNA, RIKEN full-length enriched library, c	62.8	6.20%	87	4	11	38
11,170	mousegp:MCHR17-247108|--|SWTR_MUS:Q61512_MOUSE: EGP314 precursor	35	45.10%	87	14	7	39
6,187	mousegp:MCHR9-074359|--|SWTR_MUS:TREA_MOUSE: Trehalase precursor	65.3	47%	84	24	2	40

Claims

1. Method for purifying brush border membranes comprising the following steps:
a) providing tissue comprising brush border membranes;
b) adding an inhibitor of microvillar membrane peptidases to the tissue;
c) homogenizing the tissue;
d) subjecting the homogenate to a low speed centrifugation resulting in a pellet and a supernatant;
e) subjecting the supernatant of step d) to a high speed centrifugation resulting in a pellet and a supernatant;
f) resuspending the pellet of step e);
g) subjecting the resuspended pellet of step f) to a $CaCl_2$ or $MgCl_2$ precipitation resulting in a suspension;
h) subjecting the suspension of g) to a low speed centrifugation resulting in a pellet and a supernatant;
i) subjecting then the supernatant of step h) to a high speed centrifugation resulting in a pellet and supernatant; and
k) washing the pellet of step i).

2. Method according to claim 1 wherein the peptidase inhibitor is amastatin.

3. Method according to claim 1 or 2, wherein a peptidase substrate is added in step b).

4. Method according to any one of claims 1 to 3, wherein a peptidase substrate is added in step f).

5. Method according to claim 3 or 4, wherein, the peptidase substrate is Angiotensin 1-5, Ala-Pro-PNA, or Met-Bradykinin, or a mixture thereof.

6. Method according to any one of claims 1 to 5, wherein the peptidase inhibitor is additionally added in step f).

7. Method according to any one of claims 1 to 5, wherein the peptidase inhibitor is additionally added at each one of steps c) to k) of the method.

8. Method according to any one of claims 1 to 7, wherein a chelating agent is added to the supernatant resulting from step h).

9. Method according to any one of claims 1 to 8, wherein in step k) the pellet of step i) is washed with a high salt solution and a high pH solution.

10. Purified brush border membranes obtainable by the methods according to any one of claims 1 to 9.

11. Use of amastatin for purifying brush border membranes.

12. Methods, use and proteins substantially as described herein before especially with reference to the foregoing examples.

***

Figure 1

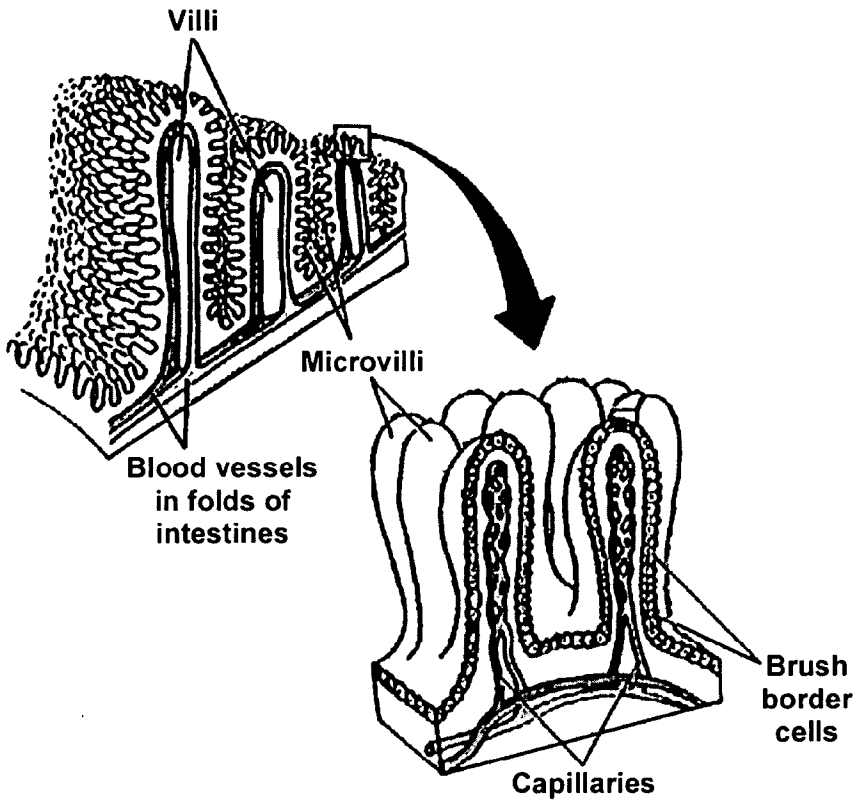

Villi
Microvilli
Blood vessels in folds of intestines
Brush border cells
Capillaries

Figure 2

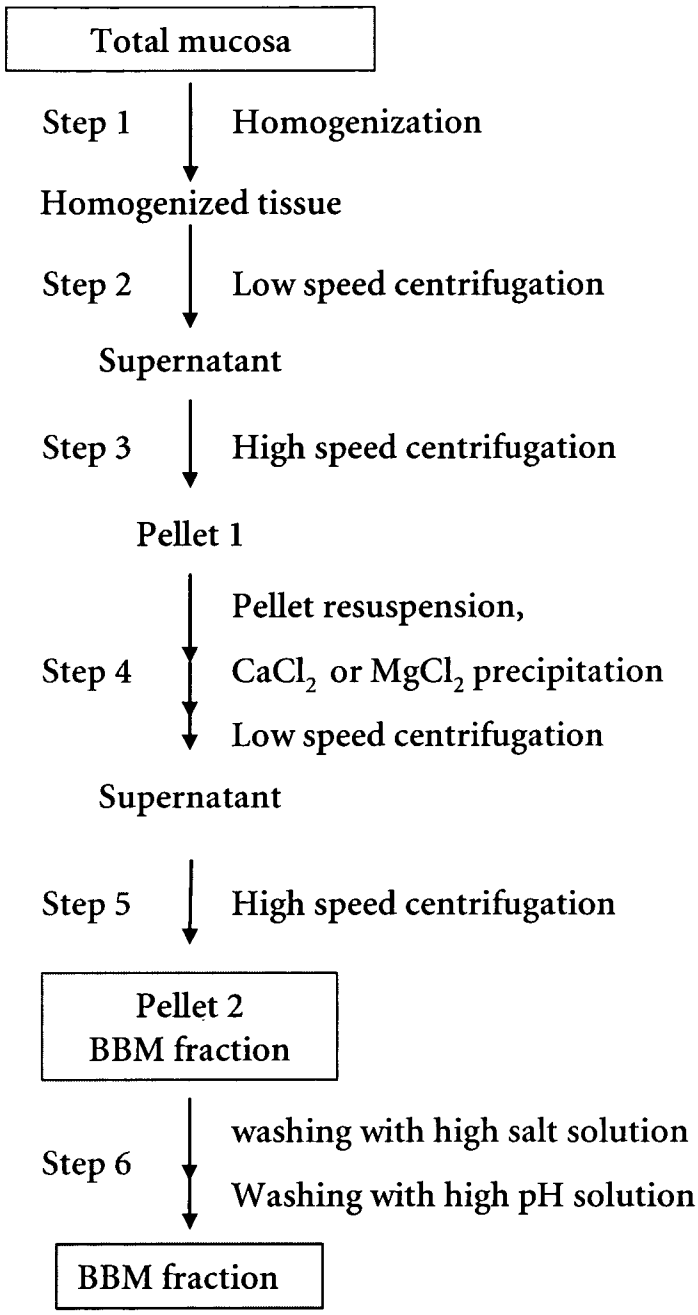

Total mucosa
Step 1
Homogenization
Homogenized tissue
Step 2
Low speed centrifugation
Supernatant
Step 3
High speed centrifugation
Pellet 1
Step 4
Pellet resuspension,
$CaCl_2$ or $MgCl_2$ precipitation
Low speed centrifugation
Supernatant
Step 5
High speed centrifugation
Pellet 2
BBM fraction
Step 6
washing with high salt solution
Washing with high pH solution
BBM fraction

Figure 3

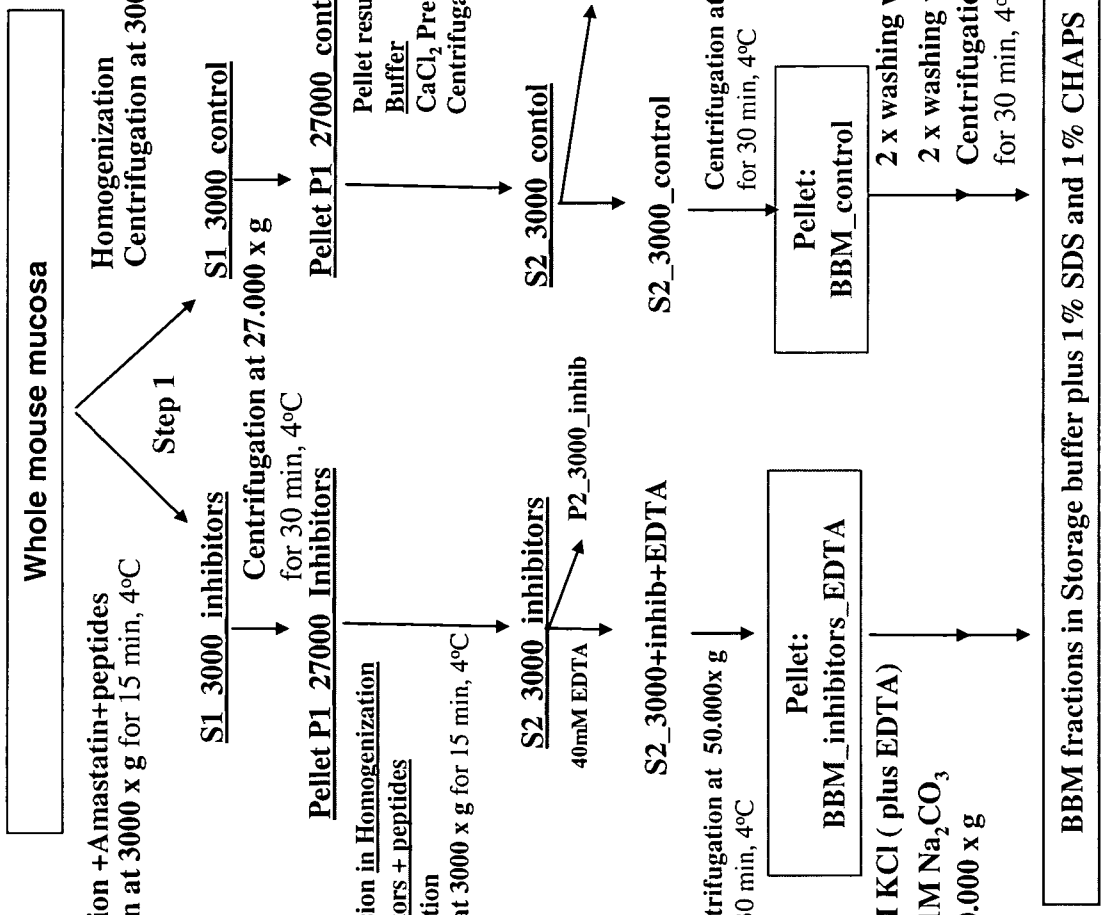

Whole mouse mucosa
Homogenization +Amastatin+peptides
Centrifugation at 3000 x g for 15 min, 4ºC
Homogenization
Centrifugation at 3000 x g for 15 min, 4ºC
Step 1
S1_3000_inhibitors
Centrifugation at 27.000 x g
for 30 min, 4ºC
S1_3000_control
Pellet P1_27000_Inhibitors
Pellet P1_27000_control
Pellet resuspension in Homogenization Buffer + Inhibitors + peptides
CaCl2 Precipitation
Centrifugation at 3000 x g for 15 min, 4ºC
Pellet resuspension in Homogenization Buffer
CaCl2 Precipitation
Centrifugation at 3000 x g for 15 min, 4ºC
S2_3000_inhibitors
40mM EDTA
P2_3000_inhib
S2_3000_contol
P3_3000 cont
S2_3000+inhib+EDTA
S2_3000_control
Centrifugation at 50.000x g
for 30 min, 4ºC
Centrifugation at 50.000x g
for 30 min, 4ºC
Pellet:
BBM_inhibitors_EDTA
Pellet:
BBM_control
2 x washing with 1M KCl (plus EDTA)
2 x washing with 0.1M Na2CO3
Centrifugation at 50.000 x g
for 30 min, 4ºC
2 x washing with 1M KCl
2 x washing with 0.1M Na2CO3
Centrifugation at 50.000 x g
for 30 min, 4ºC
BBM fractions in Storage buffer plus 1% SDS and 1% CHAPS

Figure 4:

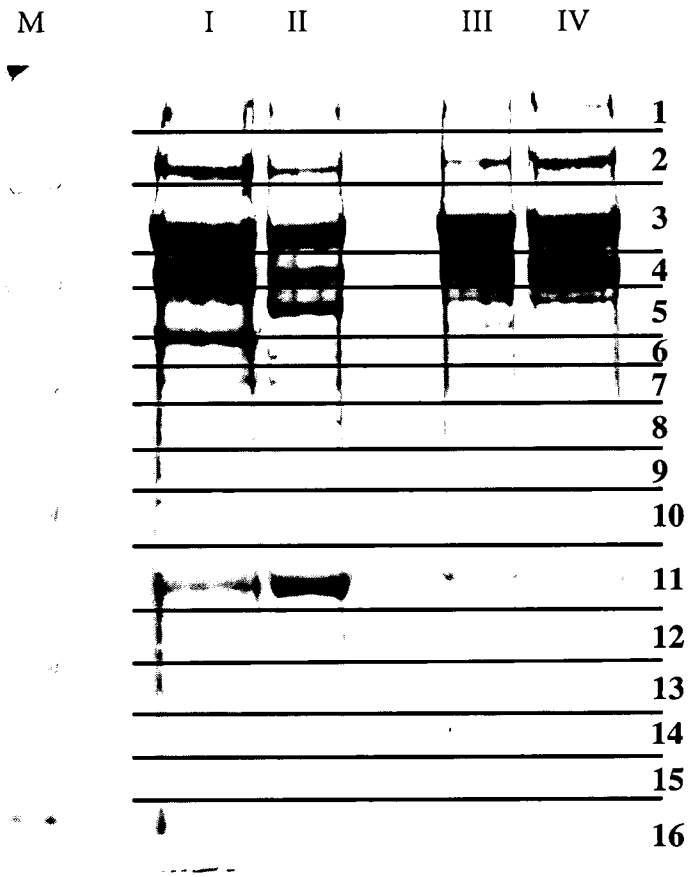
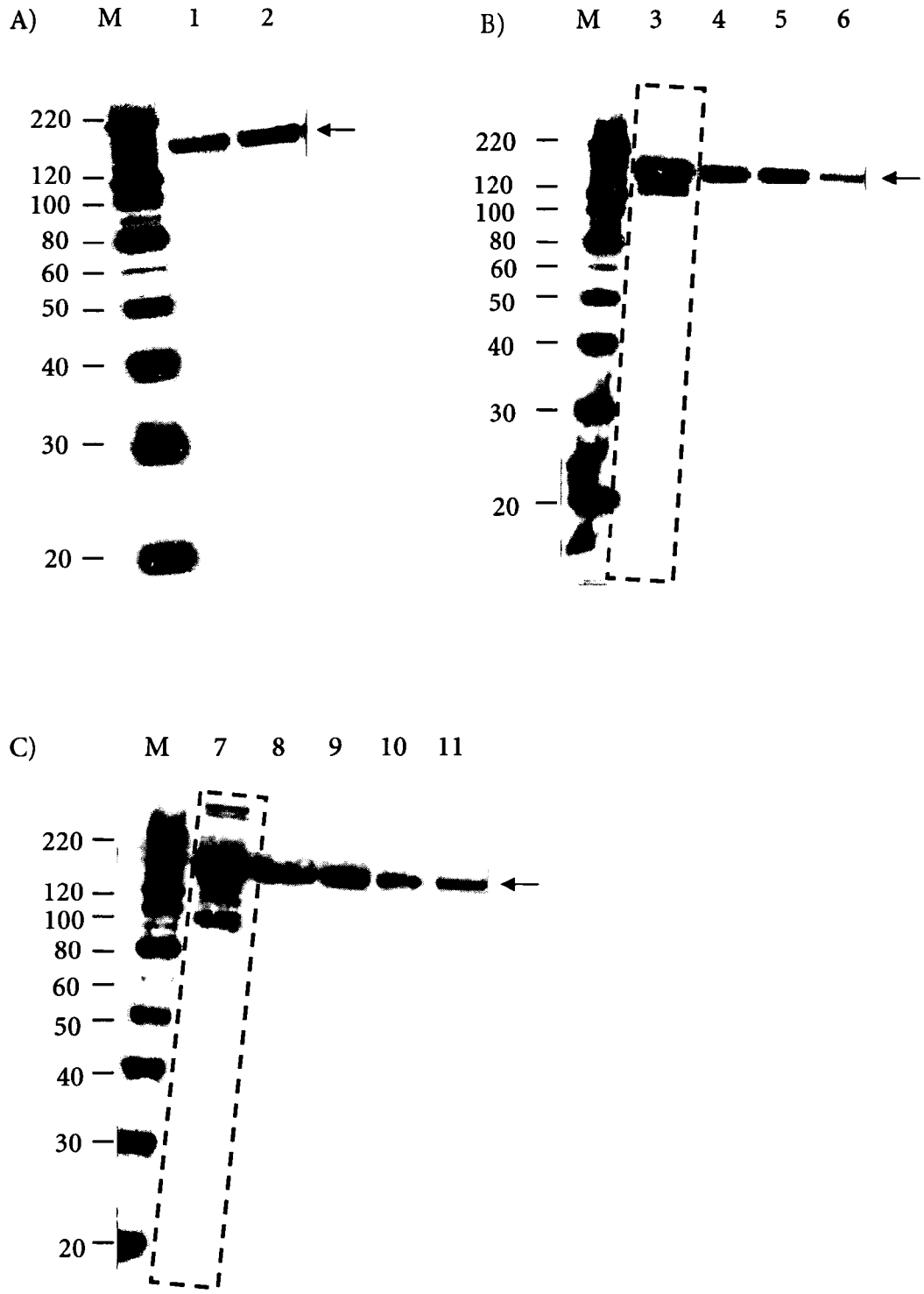


Figure 5


A)
M
1
2
220
120
100
80
60
50
40
30
20
B)
M
3
4
5
6
220
120
100
80
60
50
40
30
20
C)
M
7
8
9
10
11
220
120
100
80
60
50
40
30
20

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/003502

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/06 C07K5/02
ADD. G01N33/68

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 C07K

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 practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIAO CHUNYAN ET AL: "Purification and characterization of Cry1Ac toxin binding proteins from the brush border membrane of Helicoverpa armigera midgut." CURRENT MICROBIOLOGY DEC 2005, vol. 51, no. 6, December 2005 (2005-12), pages 367-371, XP002485571 ISSN: 0343-8651	1,3,4,6, 10
A	page 368, column 1, line 17 - line 22	2,5,7-9, 11

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 2008	05/08/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Wiame, Ilse

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/003502

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L	KNIGHT P J K ET AL: "THE RECEPTOR FOR BACILLUS THURINGIENSIS CRYLA(C) DELTA-ENDOTOXIN INTHE BRUSH BORDER MEMBRANE OF THE LEPIDOPTERAN MANDUCA SEXTA IS AMINOPEPTIDASE N" MOLECULAR MICROBIOLOGY, BLACKWELL SCIENTIFIC, OXFORD, vol. 11, no. 3, 1 January 1994 (1994-01-01), pages 429-436, XP000992663 ISSN: 0950-382X This document completes the disclosure of document XP002485571 with respect to the CHAPS-solubilisation of the BBMV. page 433, column 2, last paragraph - page 434, column 1, line 3	
X	WOLFERSBERGER M ET AL: "PREPARATION AND PARTIAL CHARACTERIZATION OF AMINO ACID TRANSPORTING BRUSH BORDER MEMBRANE VESICLES FROM THE LARVAL MIDGUT OF THE CABBAGE BUTTERFLY PIERIS-BRASSICAE" COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY A, vol. 86, no. 2, 1987, pages 301-308, XP002485572 ISSN: 0300-9629 abstract page 302, column 1, line 6 - line 55	1,6,10
X	BIBER J ET AL: "A high yield preparation for rat kidney brush border membranes. Different behaviour of lysosomal markers." BIOCHIMICA ET BIOPHYSICA ACTA 2 OCT 1981, vol. 647, no. 2, 2 October 1981 (1981-10-02), pages 169-176, XP002485570 ISSN: 0006-3002 Scheme I abstract	1,6,10
X	CHEESEMAN CHRIS I ET AL: "Isolation of intestinal brush-border membranes." CURRENT PROTOCOLS IN CELL BIOLOGY / EDITORIAL BOARD, JUAN S. BONIFACINO ... [ET AL.] APR 2006, vol. Chapter 3, April 2006 (2006-04), page Unit 3.21, XP002485573 ISSN: 1934-2616 the whole document	1,6,10

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/003502

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BABUSIAK MAREK ET AL: "Native proteomic analysis of protein complexes in murine intestinal brush border membranes" PROTEOMICS, vol. 7, no. 1, January 2007 (2007-01), pages 121-129, XP002485574 ISSN: 1615-9853 cited in the application	10
A	the whole document	1,6,7
L	ANONYMOUS: "Complete Mini Protease Inhibitor Cocktail Tablets" June 2005 (2005-06), ROCHE APPLIED SCIENCE , XP002486363 This document completes the disclosure of document XP002485574 with respect to the Roche Complete Mini protease inhibitors referred to in par. 2.1 of p. 122. the whole document	
X	ROWELL A: "The expression of intestinal sodium-glucose contransporter in cervids" ZEITSCHRIFT FUER SAEUGETIERKUNDE, URBAN UND FISCHER VERLAG, vol. 62, no. SUPPL.2, 1 January 1997 (1997-01-01), pages 204-208, XP008093570 ISSN: 0044-3468	10
A	page 205, line 24 - line 28 page 207, line 7 - line 13	1,6,7
X	DANIEL H ET AL: "Functional separation of dipeptide transport and hydrolysis in kidney brush border membrane vesicles." THE FASEB JOURNAL : OFFICIAL PUBLICATION OF THE FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY JUL 1994, vol. 8, no. 10, July 1994 (1994-07), pages 753-759, XP002485575 ISSN: 0892-6638	10
A	the whole document	2,11
X	WO 95/07930 A (CV THERAPEUTICS INC [US]; HAUSER HELMUT [CH]) 23 March 1995 (1995-03-23) the whole document	10

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 12

Claim 12 refers to the description, contrary to Rule 6.2(a) PCT, without clearly identifying which subject-matter thereof is to be considered as included in the claim. Therefore a meaningfull search is not possible for said claim.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2)PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2008/003502

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 12
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2008/003502

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9507930 A	23-03-1995	AU 7797594 A	03-04-1995