Supplemental Materials and Methods

*1.* *Animals and sensitization model design*

A total of 32 female inbred high IgE-responder Brown Norway (BN) rats (aged 4 weeks) were purchased from Charles River (Beijing, China; Permission number “SYXK (Jing) 2016-0006”). All experiments were performed in strict accordance with the NCH guidelines from China for the care and use of laboratory animals (NHC Publication No.11, Rev. 2016) and approved by the Animal Care Committee of Nanchang University (Nanchang, China). The animal sensitization protocol was performed according to previous studies (Knippels & Penninks, 2003) with some modifications. The rats were housed in cages (four rats/cage) at 22 ± 2 ℃, relative humidity was 55 ± 5 %, and a light/dark cycle of 12/12 h. Rats were randomly distributed into four groups (Control, PM, UHTM and DSM group) and each group consisted of eight rats. After a week of acclimatization, BN rats were administered by oral gavage each day for 55 days with 0.5 mL of either cow’s milk (10 mg protein, treated groups) or purified water (control group). On the 56th day, each rat was dosed by gavage with 1 mL of either cow’s milk (30 mg protein, treated groups) or purified water (control group), and 30 min later, blood and intestine were collected. For serum separation, blood was clotted overnight at 4 ℃ and serum was separated with centrifugation at 3500 rpm for 10 minutes at 4 °C and stored at -80 ℃ for further analysis. Figure S1 is an overview of the animal experimental design.

*2. The detection of specific antibody by indirect ELISA*

Specific IgE, IgG, IgG1, and IgG2a responses to cow’s milk proteins were performed by indirect ELISAs. Microtitre plates (96-well, Corning, NY, USA) were coated with 2 μg/mL of allergen (100 μL/well) diluted in 0.05 M carbonate buffer (15 mmol/mL Na2CO3, 35 mmol/mL NaHCO3, pH 9.6) and incubated overnight at 4 °C. Plates were washed three times by PBS with 0.01% Tween 20 (PBS-T). Those plates were blocked with 250 μL/well of 3% gelatin in PBS for 1 h at 37 °C. Dilutions of serum was 1:10, 1:40,000, 1:8,000, and 1:8,000 for testing IgE, IgG, IgG1 and IgG2a, respectively. The diluted sera (100 μL/well) were added and incubated for 1 h at 37℃. For detection IgG, IgG1, and IgG2a, 100 μL of HRP-labelled mouse-anti-rat IgG, IgG1, and IgG2a (Southern Biotech, Birmingham, AL, USA) diluted 1: 5,000 (v:v) in PBS was added to each well and incubated for 1 h at 37℃, respectively. For the IgE binding experiments, 100 μL of biotin-labelled mouse-anti-rat IgE (Bio-Rad, California, USA) diluted 1: 5,000 (v:v) in PBS was added to each well and incubated for 1 h at 37℃, and after washing, streptavidin-HRP (NeoBioscience, Shanghai, China) diluted 1:60 (v:v) in PBS was added to each well. The step for visualization of the specific antibody response was same as the above.

**Table S1**. Formulation of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Constituent (mol/L) | | | | | |
| KCl (0.5) | KH2PO4 (0.5) | NaHCO3 (1) | NaCl (2) | MgCl2 (0.15) | (NH4)2CO3 (0.5) |
| gastric stock electrolyte solution (mL) | 6.9 | 0.9 | 12.5 | 11.8 | 0.4 | 0.5 |
| Added water to 400mL | | | | | |
| intestinal stock electrolyte solution (mL) | 6.8 | 0.8 | 42.5 | 9.6 | 1.1 |  |
| Added water to 500mL | | | | | |

**Table S2.** Clinical data of 10 individuals with cow’s milk allergy

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Number | Sex | Age | Clinical symptoms | Specific IgE level (KUA/L) |
| 1 | Female | 10 months old | Asthmatic Bronchial Pneumonia | 3.5 |
| 2 | Male | 4 months old | ND\* | 2.7 |
| 3 | Male | 1 months old | Bronchial Asthma | 2.9 |
| 4 | Female | 13 months old | Pneumonia | 1.0 |
| 5 | Female | 4 years old | Allergic Rhinitis | 3.2 |
| 6 | Male | 4 months old | Urticaria | 5.9 |
| 7 | Male | 9 months old | Urticaria | 56.8 |
| 8 | Male | 11 months old | ND\* | 18.0 |
| 9 | Male | 1 year old | Leukocytosis | 43,7 |
| 10 | Male | 3 years old | Allergic Rhinitis | 3.7 |

ND\*—Not Done

**Table S3**. Formulation of Kreb’s solution

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Constituent (mol/L) | | | | | | |
| KCl (0.47) | KH2PO4 (120) | NaHCO3 (0.248) | NaCl (1.17) | MgCl2 (0.1) | CaCl2(0.256) (0.256CO3 (0.5) | Glucose |
| Kreb’s solution (mL) | 10 | 10 | 100 | 100 | 12 | 10 | 2g |
| Added water to 1L | | | | | | |

Table S4. The amino acid sequence information of synthetic peptides.

|  |  |  |
| --- | --- | --- |
| Peptide | Amino acid sequence | Purity |
| P1(β- LG -AA41-60) | VYVEELKPTPEGDLEILLQK | 95% |
| P2(β-LG -AA61-69) | WENGECAQK | 95% |
| P3(β-LG -AA76-83) | TKIPAVFK | 95% |
| P4(β-LG -AA84-91) | IDALNENK | 95% |
| P5(β-LG -AA92-100) | VLVLDTDYK | 95% |
| P6(β-LG -AA125-135) | TPEVDDEALEK | 95% |
| P7(β-LG -AA125-138) | TPEVDDEALEKFDK | 95% |
| P8(β-LG -AA142-148) | ALPMHIR | 95% |
| P9(α-LA -AA63-79) | DDQNPHSSNICNISCDK | 95% |
| P10(α-LA -AA80-93) | FLDDDLTDDIMCVK | 95% |
| P11(α-LA -AA99-108) | VGINYWLAHK | 95% |
| P12(α-LA -AA115-122) | LDQWLCEK | 95% |
| P13(αS1-casein -AA84-90) | EDVPSER | 95% |
| P14(αS1-casein -AA125-132) | EGIHAQQK | 95% |
| P15(αS2-casein -AA25-32) | NMAINPSK | 95% |
| P16(αs2-casein -AA71-76) | ITVDDK | 95% |

**Table S5**. The remaining peptides derived from the pasteurized milk (PM), ultra-heat-treated milk (UHTM) and dried skim milk (DSM) after simulating gastrointestinal digestion were identified by nano-LC-QE.

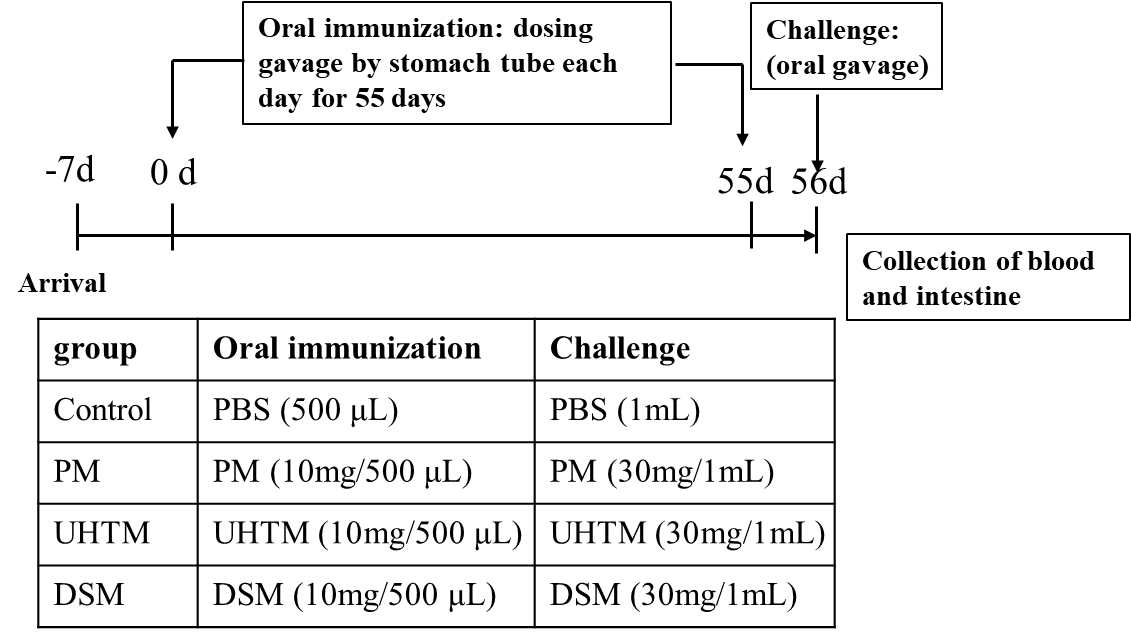
|  |  |  |
| --- | --- | --- |
| Measured MW (MH+) | Identification | Sequence |
| PM group |  |  |
| 2707.37 | β-LG (15-40) | VAGTWYSLAMAASDISLLDAQSAPLR |
| 2313.25 | β-LG (41-60) | VYVEELKPTPEGDLEILLQK |
| 903.56 | β-LG (76-83) | TKIPAVFK |
| 916.47 | β-LG (84-91) | IDALNENK |
| 1065.58 | β-LG (92-100) | VLVLDTDYK |
| 1193.67 | β-LG (92-101) | VLVLDTDYKK |
| 1635.77 | β-LG (125-138) | TPEVDDEALEKFDK |
| 837.47 | β-LG (142-148) | ALPMHIR |
| 1699.94 | α-LA (95-108) | ILDKVGINYWLAHK |
| 1200.65 | α-LA (99-108) | VGINYWLAHK |
| 1759.94 | αS1-casein (8-22) | HQGLPQEVLNENLLR |
| 1337.67 | αS1-casein (80-90) | HIQKEDVPSER |
| 1267.70 | αS1-casein (91-101) | YLGYLEQLLR |
| 946.52 | αS1-casein (35-42) | EKVNELSK |
| 223.23 | αS1-casein (4-22) | HPIKHQGLPQEVLNENLLR |
| 1384.72 | αS1-casein (23-34) | FFVAPFPEVFGK |
| 1237.66 | αS1-casein (24-34) | FVAPFPEVFGK |
| 875.53 | αS1-casein (103-109) | KYKVPQL |
| 910.47 | αS1-casein (125-132) | EGIHAQQK |
| 874.44 | αS2-casein (25-32) | NMAINPSK |
| 1367.9 | αS2-casein (81-91) | ALNEINQFYQK |
| 1246.64 | αS2-casein (71-80) | ITVDDKHYQK |
| 1195.67 | αS2-casein (115-125) | NAVPITPTLNR |
| 1247.66 | αS2-casein (151-160) | TKLTEEEKNR |
| 979.56 | αS2-casein (174-181) | FALPQYLK |
| 1137.55 | β-casein (114-122) | YPVEPFTER |
| 1151.69 | β-casein (199-209) | GPVRGPFPIIV |
| 1013.52 | β-casein (106-113) | HKEMPFPK |
| 780.49 | β-casein (170-176) | VLPVPQK |
| 1512.78 | β-casein (46-58) | QDKIHPFAQTQSL |
| 1242.68 | κ-casein (76-86) | WQVLSNTVPAK |
| 1269.61 | κ-casein (1-10) | QEQNQEQPIR |
| UHTM group |  |  |
| 2707.37 | β-LG (15-40) | VAGTWYSLAMAASDISLLDAQSAPLR |
| 2313.25 | β-LG (41-60) | VYVEELKPTPEGDLEILLQK |
| 903.56 | β-LG (76-83) | TKIPAVFK |
| 1256.72 | β-LG (83-93) | KIDALNENKVL |
| 916.47 | β-LG (84-91) | IDALNENK |
| 1193.67 | β-LG (92-101) | VLVLDTDYKK |
| 1065.58 | β-LG (92-100) | VLVLDTDYK |
| 1245.58 | β-LG (125-135) | TPEVDDEALEK |
| 1635.77 | β-LG (125-138) | TPEVDDEALEKFDK |
| 1699.94 | α-LA (95-108) | ILDKVGINYWLAHK |
| 1200.65 | α-LA (99-108) | VGINYWLAHK |
| 1759.94 | αS1-casein (8-22) | HQGLPQEVLNENLLR |
| 1237.66 | αS1-casein (24-34) | FVAPFPEVFGK |
| 1337.67 | αS1-casein (80-90) | HIQKEDVPSER |
| 946.52 | αS1-casein (35-42) | EKVNELSK |
| 1267.70 | αS1-casein (91-101) | YLGYLEQLLR |
| 910.47 | αS1-casein (125-132) | EGIHAQQK |
| 1506.77 | αS1-casein (120-132) | LHSMKEGIHAQQK |
| 1386.64 | αS2-casein (138-149) | TVDMESTEVFTK |
| 1247.65 | αS2-casein (151-160) | TKLTEEEKNR |
| 979.56 | αS2-casein (174-181) | FALPQYLK |
| 1246.64 | αS2-casein (71-80) | ITVDDKHYQK |
| 1195.67 | αS2-casein (115-125) | NAVPITPTLNR |
| 1351.78 | αS2-casein (114-125) | RNAVPITPTLNR |
| 1137.55 | β-casein (114-122) | YPVEPFTER |
| 1981.86 | β-casein (33-48) | FQSEEQQQTEDELQDK |
| 1591.93 | β-casein (170-183) | VLPVPQKAVPYPQR |
| 1458.77 | β-casein (193-205) | YQEPVLGPVRGPF |
| 2682.32 | β-casein (141-163) | QSWMHQPHQPLPPTVMFPPQSVL |
| 1242.68 | κ-casein (76-86) | WQVLSNTVPAK |
| DSM group |  |  |
| 2313.25 | β-LG (41-60) | VYVEELKPTPEGDLEILLQK |
| 903.56 | β-LG (76-83) | TKIPAVFK |
| 1256.72 | β-LG (83-93) | KIDALNENKVL |
| 1193.67 | β-LG (92-101) | VLVLDTDYKK |
| 1065.58 | β-LG (92-100) | VLVLDTDYK |
| 1243.61 | β-LG (123-132) | VRTPEVDDEAL |
| 1245.58 | β-LG (125-135) | TPEVDDEALEK |
| 1635.77 | β-LG (125-138) | TPEVDDEALEKFDK |
| 1759.94 | αS1-casein (8-22) | HQGLPQEVLNENLLR |
| 1384.72 | αS1-casein (23-34) | FFVAPFPEVFGK |
| 1337.67 | αS1-casein (80-90) | HIQKEDVPSER |
| 1267.70 | αS1-casein (91-101) | YLGYLEQLLR |
| 946.52 | αS1-casein (35-42) | EKVNELSK |
| 2021.01 | αS1-casein (125-142) | EGIHAQQKEPMIGVNQEL |
| 690.36 | αS2-casein (71-76) | ITVDDK |
| 1246.64 | αS2-casein (71-80) | ITVDDKHYQK |
| 1367.69 | αS2-casein (81-91) | ALNEINQFYQK |
| 1379.66 | αS2-casein (126-137) | EQLSTSEENSKK |
| 1251.57 | αS2-casein (126-136) | EQLSTSEENSK |
| 1386.64 | αS2-casein (138-149) | TVDMESTEVFTK |
| 1247.65 | αS2-casein (151-160) | TKLTEEEKNR |
| 979.56 | αS2-casein (174-181) | FALPQYLK |
| 1195.67 | αS2-casein (115-125) | NAVPITPTLNR |
| 1351.78 | αS2-casein (114-125) | RNAVPITPTLNR |
| 874.44 | αS2-casein (40-47) | NMAINPSK |
| 1137.55 | β-casein (114-122) | YPVEPFTER |
| 1981.86 | β-casein (33-48) | FQSEEQQQTEDELQDK |
| 1013.52 | β-casein (106-113) | HKEMPFPK |
| 1591.92 | β-casein (170-183) | VLPVPQKAVPYPQR |
| 889.48 | β-casein (98-105) | VKEAmAPK |
| 1512.78 | β-casein (46-58) | QDKIHPFAQTQSL |
| 1242.68 | κ-casein (76-86) | WQVLSNTVPAK |

**Table S6**. Identification by nanoESI-MSMS of pasteurized milk (PM), ultra-heat-treated milk (UHTM) and dried skim milk (DSM) derived peptides surviving *in vitro* transepithelial transport by sensitized rats’ intestines.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Measured MW (MH+) | Identification | | Sequence | |
| PM group |  | |  | |
| 2313.25 | β-LG (41-60) | | VYVEELKPTPEGDLEILLQK | |
| 1179.47 | β-LG (61-69) | | WENGECAQK | |
| 701.45 | β-LG (70-75) | | KIIAEK | |
| 674.42 | β-LG (76-83) | | TKIPAVFK | |
| 916.47 | β-LG (84-91) | | IDALNENK | |
| 1065.58 | β-LG (92-100) | | VLVLDTDYK | |
| 1193.67 | β-LG (92-101) | | VLVLDTDYKK | |
| 1635.77 | β-LG (125-138) | | TPEVDDEALEKFDK | |
| 837.47 | β-LG (142-148) | | ALPMHIR | |
| 1715.80 | β-LG (149-162) | | LSFNPTQLEEQCHI | |
| 1699.75 | α-LA (80-93) | | FLDDDLTDDIMCVK | |
| 1200.65 | | α-LA (99-108) | VGINYWLAHK |
| 1091.51 | | α-LA (115-122) | LDQWLCEK |
| 831.38 | | αS1-casein (84-90) | EDVPSER |
| 874.44 | | αS2-casein (25-32) | NMAINPSK |
| UHTM group |  | |  | |
| 1179.47 | β-LG (61-69) | | WENGECAQK | |
| 674.42 | β-LG (78-83) | | IPAVFK | |
| 1065.58 | β-LG (92-100) | | VLVLDTDYK | |
| 1193.67 | β-LG (92-101) | | VLVLDTDYKK | |
| 1245.58 | β-LG (125-135) | | TPEVDDEALEK | |
| 1635.77 | β-LG (125-138) | | TPEVDDEALEKFDK | |
| 837.47 | β-LG (142-148) | | ALPMHIR | |
| 1715.80 | β-LG (149-162) | | LSFNPTQLEEQCHI | |
| 2003.81 | | α-LA (63-79) | DDQNPHSSNICNISCDK |
| 1200.65 | | α-LA (99-108) | VGINYWLAHK |
| 1699.75 | | α-LA (80-93) | FLDDDLTDDIMCVK |
| 1091.51 | | α-LA (115-122) | LDQWLCEK |
| 831.38 | αS1-casein (84-90) | | EDVPSER | |
| 9910.47 | αS1-casein (125-132) | | EGIHAQQK | |
| 874.44 | αS2-casein (25-32) | | NMAINPSK | |
| 690.36 | αS2-casein (71-76) | | ITVDDK | |
| DSM group |  | |  | |
| 674.42 | β-LG (78-83) | | IPAVFK | |
| 1065.58 | β-LG (92-100) | | VLVLDTDYK | |

Table S7. Identification by nanoESI-MSMS of the pasteurized milk (PM), ultra-heat-treated milk (UHTM) and dried skim milk (DSM) derived peptides surviving *in vitro* transepithelial transport by rat’s intestine from the control group

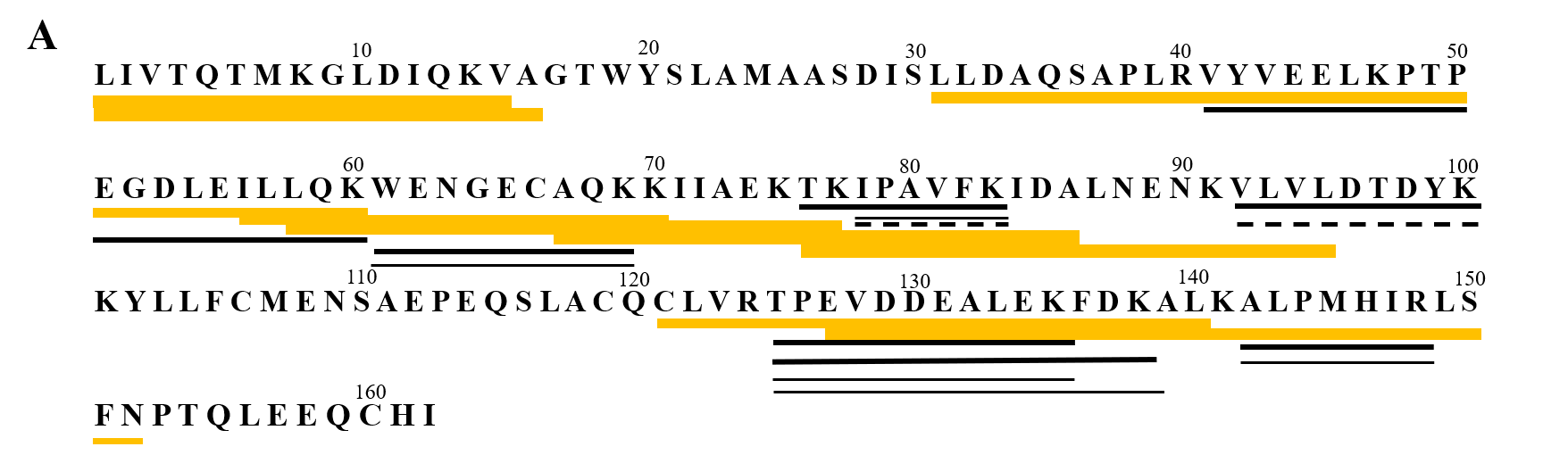
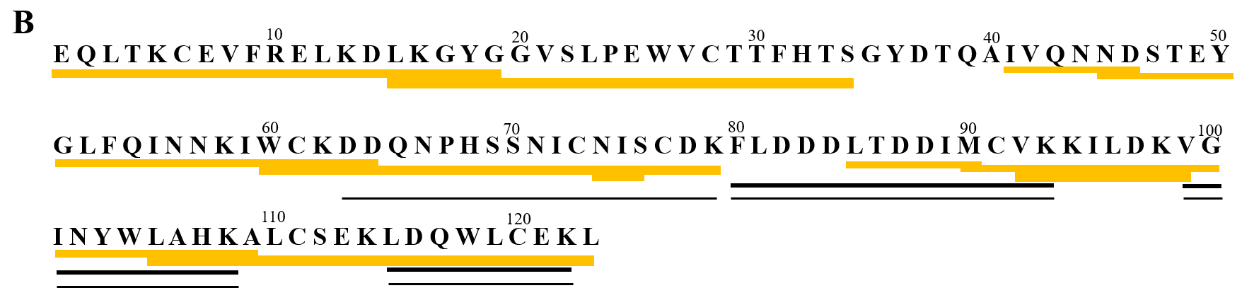
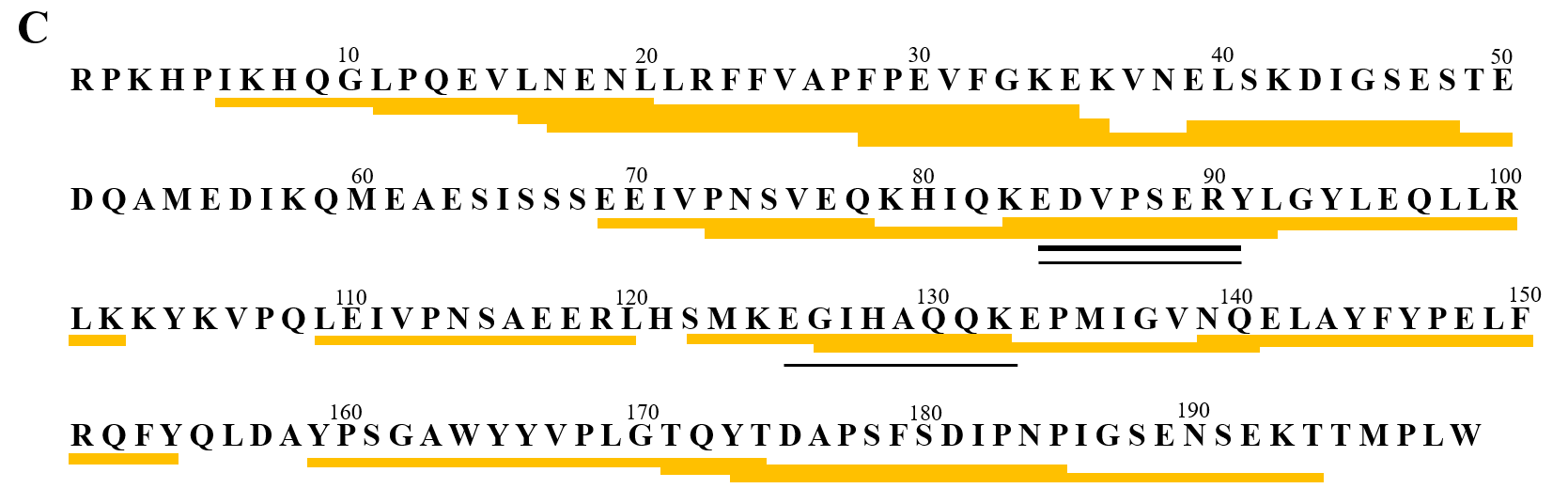
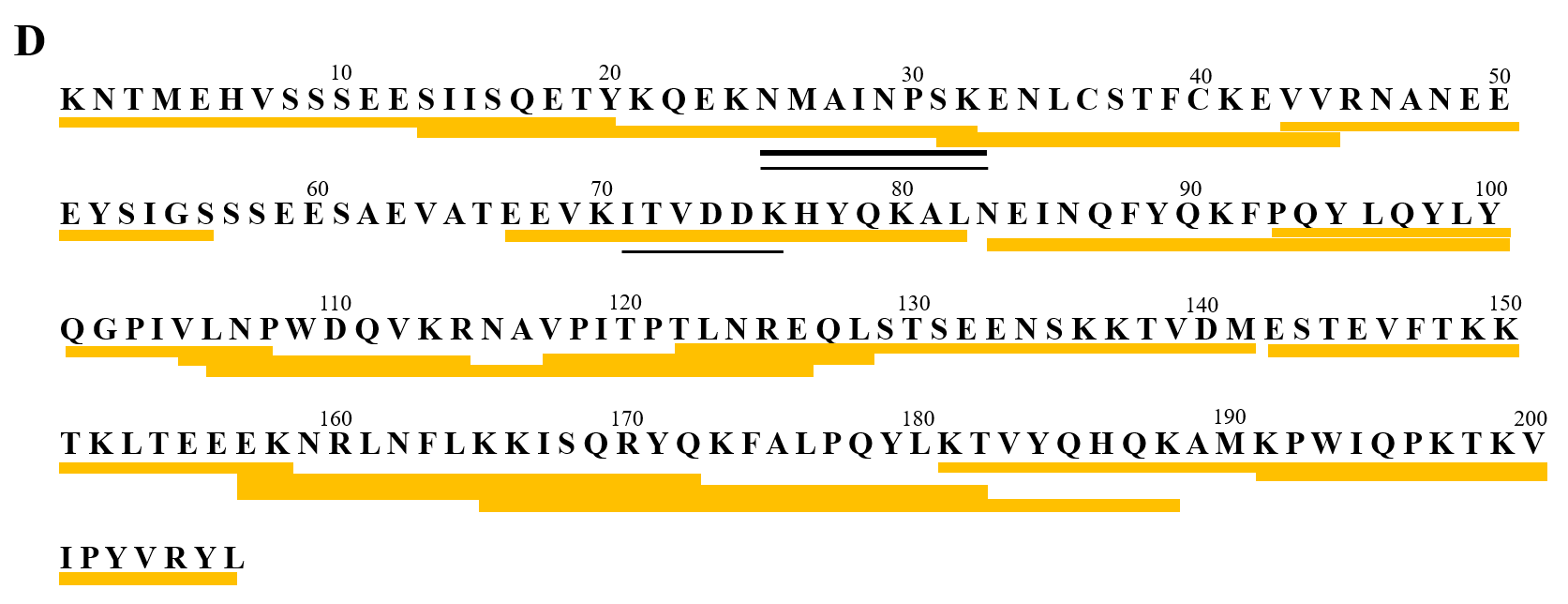
|  |  |  |
| --- | --- | --- |
| Measured MW (MH+) | Identification | Sequence |
| PM group |  |  |
| 2313.25 | β-LG (41-60) | VYVEELKPTPEGDLEILLQK |
| 1179.47 | β-LG (61-69) | WENGECAQK |
| 674.42 | β-LG (78-83) | IPAVFK |
| 916.47 | β-LG (84-91) | IDALNENK |
| 1245.58 | β-LG (125-135) | TPEVDDEALEK |
| 1715.80 | β-LG (149-162) | LSFNPTQLEEQCHI |
| UHTM group |  |  |
| 674.42 | β-LG (78-83) | IPAVFK |
| 1384.73 | αS1- casein (23-34) | FFVAPFPEVFGK |
| DSM group |  |  |
| 674.42 | β-LG (78-83) | IPAVFK |



**Figure S1**. Sensitization experiments. Treatment project: Brown Norway rats were administered by oral gavage each day for 56 days with PBS, pasteurized milk (PM), ultra-heat-treated milk (UHTM) and dried skim milk (DSM), respectively. The experimental setup with n=8 rats per group is shown in the insert: Control group, PM group, UHTM group, DSM group.



**Figure S2.** The levels of specific IgE/IgG/ IgG2a/IgG1 in sera from rat immunized by different cow’s milk sample including pasteurized milk (PM), ultra-heat-treated milk (UHTM) and dried skim milk (DSM). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 indicate significant difference as compared to that of control group.



**Figure S3.** The peptides were identified by LC-MS/MS at the end of β-LG (A), α-LA (B), αS1-casein (C), and αS2-casein (D) gastrointestinal digestion followed by intestinal transportation. The reported IgE linear epitopes were represented as the yellow bars. Peptides found in pasteurized milk (PM), ultra-heat-treated milk (UHTM) and dried skim milk (DSM) were represented as thick black bars, thin black bars and dashed lines, respectively.

Reference

Knippels, L. M., & Penninks, A. H. (2003). Assessment of the allergic potential of food protein extracts and proteins on oral application using the brown Norway rat model. Environ Health Perspect, 111 (2), 233-238.