

Universal artificial hapten and universal artificial complete antigen for capsaicinoids and application thereof

Field of the Invention

The present invention relates to universal artificial hapten and universal artificial complete antigen for capsaicinoids and application thereof, which belongs to the technical field of immune chemical technology.

Background

Capsaicinoids are the main chemical substances to be spicy stimuli in capsicum, wherein capsaicinoids mainly comprise over 20 known compounds of similar structure such as capsaicin, dihydrocapsaicin, homodihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and so on, wherein capsaicin and dihydrocapsaicin are about 90 % of total capsaicinoids, and provide about 90 % of spicy stimulation. With the improvement of people's living standards, the nutritional quality and medicinal value of capsaicinoids are gradually discovered. Besides capsaicinoids are widely used in food processing fields, capsaicinoids are used in fields of health care, biological material, biological weapon and other. In food processing fields, there is no a simple, effective and accurate method to distinguish the spicy degree at present. Synthetic capsaicin has the similar structure and function with natural capsaicinoids, and has more advantages in price and spicy degree than natural capsaicinoids, which are generally used as substitute for capsaicinoids. Therefore, the determination of capsaicinoids in food is of great significance to the development and standardization and quality evaluation thereof of capsicum products.

At present, the detection methods with modern instrument like high performance liquid chromatography, chromatography-mass spectrometry are the most commonly used detection methods for capsaicinoids, which are sensitive and accurate, , but the instrument is expensive, requirement of experimental environment is high, and professional operator is needed, it is difficult to achieve quick detection. In recent years, rapidly developed immunoassay method overcomes the shortcomings of the above methods, which has advantages such as high specificity, high sensitivity, large analytical capacity, quick and easy, low cost, suitable for on-site batch detections and so on, which is widely used in medicine, agriculture and other fields.

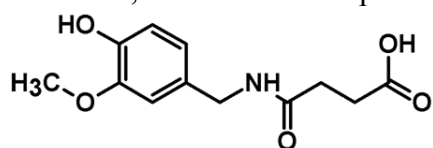
Immunoassay is an analytical method based on the specific recognition of antigen and antibody, that the core reagent is high-quality specific antibody. But the capsaicinoids have a molecular weight lower than 500, which are small molecules, which are not immunological antigenic, need to couple with macromolecular carrier protein to be immunological antigenic. Therefore, in order to obtain high-quality specific antibody, capsaicinoids were coupled with carrier protein at first to obtain immunogenic universal artificial complete antigen for capsaicinoids. At present, there are few reports related to universal artificial complete antigen for capsaicinoids at home and abroad. Therefore, the developments of universal artificial hapten and universal artificial complete antigen for capsaicinoids, not only fills up the blank of immunodetection for capsaicinoids, but also finds a new way for rapid detection for capsaicinoids, are of significance to achieve the quantitative immunodetection for the total amount of capsaicin, dihydrocapsaicin, and synthetic capsaicin.

Summary of the Invention

The object of the present invention is to provide a new universal artificial hapten and universal artificial complete antigen for capsaicinoids and application thereof.

In order to solve the above technical problems, the technical solutions adopted by the present invention comprise:

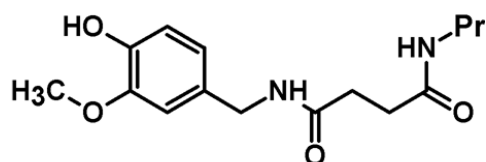
A universal artificial hapten compound for capsaicinoids is provided by the present invention, wherein the compound has the molecular structure as shown in formula I:



I

which chemical name is 4-[(4-hydroxy-3-methoxybenzyl) amino]-4-oxobutanoic acid) .

A universal artificial complete antigen compound for capsaicinoids is provided, wherein the compound has the molecular structure as shown in formula II:

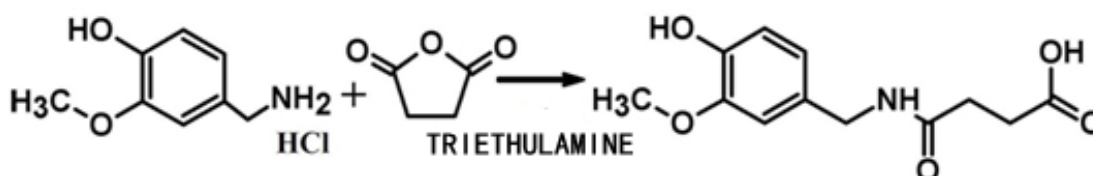


II

, Pr is carrier protein.

According to the above solutions, wherein the carrier protein is bovine serum albumin BSA, ovalbumin OVA or keyhole limpet hemocyanin KLH.

A process for synthesizing the above universal artificial hapten compound for capsaicinoids, wherein comprising synthesizing the artificial hapten compound of formula I by one step amidation reaction in the presence of triethylamine as acid-binding agent with vanillylamine hydrochloride and succinic anhydride as raw materials, the synthetic route is as follows:



According to the above solutions, wherein the process for synthesizing the above artificial hapten compound for capsaicinoids comprises the steps below: vanillylamine hydrochloride and triethylamine at a molar ratio of 1 : (0.9 to 1.2) are dissolved in tetrahydrofuran, stirred at room temperature for 20-40min, after equal molar ratio of succinic anhydride is added, reacted under stirred at room temperature for more than 7 hours, to obtain universal artificial hapten compounds I for capsaicinoids by post treatment.

According to the above solutions, wherein the post treatment comprises: after ethyl acetate is added, stirred at room temperature, filtered to obtain white precipitate which is the sample.

The process for preparing the above universal artificial complete antigen compound for capsaicinoids comprises: the universal artificial hapten for capsaicinoids is activated by N,N'-dicyclohexylcarbodiimide DCC and N-hydroxysuccinimide NHS, and then coupled with the carrier protein to obtain the universal artificial complete antigen compound.

The process for preparing the above universal artificial complete antigen compound for capsaicinoids comprises the specific steps below:

(1) Activation of universal artificial hapten for capsaicinoids: universal artificial hapten for capsaicinoids is reacted with N-hydroxysuccinimide (NHS) at room temperature for 30min-2h in N,N-dimethylformamide, then dicyclohexylcarbodiimide in N,N-dimethylformamide solution is added, reacted at room temperature for 4-6h, standing overnight at 4℃, got supernatant which is the activated artificial hapten for capsaicinoids, wherein the molar ratios of N-hydroxysuccinimide (NHS) to dicyclohexylcarbodiimide are 1:(1-1.2);

or artificial hapten for capsaicinoids is dissolved in N,N-dimethylformamide, after tributylamin and isobutyl chloroformate are added sequentially, reacted at room temperature for 0.5-3h, got reaction solution which is the activated artificial hapten for capsaicinoids, wherein the molar ratios of tributylamin to isobutyl chloroformate are 1:(1-1.2);

(2) The supernatant obtained in the above step (1) which is the activated universal artificial hapten for capsaicinoids is added dropwise into the phosphate buffer with carrier protein, wherein carrier protein has the concentration greater than or equal to 2mg/mL, reacted at room temperature for 4-8h, whereby: wherein the molar ratio of activated universal artificial hapten for capsaicinoids to carrier protein is great than 10:1, then dialyzed to obtain universal artificial complete antigen for capsaicinoids.

According to the above solutions, wherein the phosphate buffer with carrier protein was obtained by dissolved the carrier protein in 0.2M pH8.0 phosphate buffer solution.

According to the above solutions, wherein dialysate was dialyzed by 0.01M PBS buffer for 72h, during the period, replaced the dialysate for every 4-8h.

The use of the above universal artificial complete antigen compound for capsaicinoids in the preparation of specific antibody for capsaicinoids. The method of the use comprises: the japanese big ear rabbits are immunized with the above universal artificial complete antigen for capsaicinoids, after immunized for 4-6 times, the rabbits are collected blood from carotid artery thereof to death, after the collected blood is centrifuged, antiserum is purified by caprylic acid-ammonium sulfate method

to obtain universal antibody for capsaicinoids, which is the globulin can have specific immune response with capsaicinoids.

The present invention has the advantages of:

According to the present invention there is provided a artificial hapten by modifying the phenolic hydroxyl of vanillylamide in capsaicin, wherein the artificial hapten can not only remain the characteristic structure (vanillylamide and partial aliphatic chain groups in molecular structure of capsaicin) of capsaicinoids to the maximum degree but also be used as an antigenic determinant, also have the active groups that can be coupled with carrier protein. Thus high affinity, high sensitivity, high specificity antibody for capsaicin can be obtained by immunized. The experimental result shows that antiserum which was obtained from the antigen immune animal according to the present invention can reach a titer of 128000, the half maximal inhibitory concentration IC_{50} of the obtained antibody for capsaicinoids was $0.7\mu\text{g/mL}$, the half maximal inhibitory concentration IC_{50} for capsaicin, dihydrocapsaicin and synthetic capsaicin were, in order, $1.1\mu\text{g/mL}$, $0.5\mu\text{g/mL}$, $1.8\mu\text{g/mL}$. It is indicated that the antiserum could specific react with capsaicinoids and could be used as universal antibody for capsaicinoids. The antigen or antibody in the present invention can be used to establish enzyme-linked immunosorbent assay, rapid tests based on colloidal gold test strip and time-resolved fluorescence test strip, to provide the key technical support to the standardized management of food spicity.

Brife Description of the Drawing

Fig. 1 shows the ultraviolet spectrogram of the universal artificial complete antigen for capsaicinoids in the present invention.

Fig. 2 shows the ultraviolet spectrogram of the universal artificial complete antigen-coating antigen for capsaicinoids in the present invention.

Detailed Description of the Invention and Specific embodiments

The test methods used in the following examples are conventional methods unless otherwise stated.

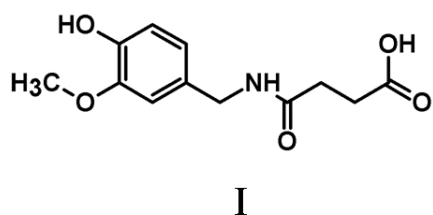
The materials, reagents and so on that are used in the following examples are commercially available unless otherwise stated.

Examples

Example 1 Preparation of the universal artificial hapten for capsaicinoids

0.28g of vanillylamine hydrochloride was weighed and dissolved in 6mL of tetrahydrofuran, 0.15g of triethylamine was added dropwise with stirring, stirred at room temperature for 30min. 0.15g (0.0015mol) of succinic anhydride was accurately weighed and added into the above reaction solution, stirred at room temperature overnight. 3mL of ethyl acetate was added into the reaction solution with stirring at room temperature for 2min, the precipitate which was obtained after filtration was hapten, which was 4-[(4-hydroxy-3-methoxybenzyl) amino]-4-oxobutanoic acid, the formula was $C_{12}H_{15}NO_5$.

The result of NMR identification was: 1H NMR (400 MHz, DMSO) δ 12.17 (s, 1H), 8.87 (s, 1H), 8.31 (d, J = 6.0 Hz, 1H), 4.21 (d, J = 5.8 Hz, 2H), 3.80 (s, 3H), 2.52 (t, J = 6.9 Hz, 2H), 2.42 (t, J = 6.7 Hz, 2H). The result was consistent with the theoretical value, which was indicated that the hapten compound was successfully synthesized.



Example 2 Preparation of the universal artificial complete antigen-immunological antigen for capsaicinoids

20.3mg (about 0.08mmol) of the above universal artificial hapten for capsaicinoids and 11.6mg (about 0.1mmol) of NHS were weighed into reaction flask, 400 μ L of DMF was added and dissolved in the reaction flask, stirred at room temperature for 30 min, 20.6mg (about 0.1mmol) of DCC was weighed and dissolved into 100 μ L of DMF, DCC/DMF solutions were added dropwise into the above reaction flask, stirred at room temperature for 4h, standing at 4°C overnight. 8000rpm/5min, the supernatant active ester solution was obtained.

The supernatant active ester solution was added dropwise into 6 mL 7mg/ml of BSA solution to react, the reaction buffer was 0.2M pH8.0 phosphate buffer. The reaction was carried out under magnetically stirred at room temperature for 4h. The reaction

solution was placed in dialysate bag, stirred and dialyzed in 0.01M pH7.4 PBS at 4°C, and changed dialysate solution every 4h, dialyzed for 72h in total. The artificial antigen-immunological antigen for capsaicin was obtained. Ultraviolet-visible spectrum of continuous scanning spectra is shown in Figure 1. The identification result shows that the artificial antigen is successfully coupled.

Example 3 Preparation of universal artificial complete antigen-coating antigen for capsaicinoids

4.55mg of the above universal artificial hapten for capsaicinoids was weighed and dissolved in 200 μ L of anhydrous DMF, then 4.27 μ L of tributylamine and 2.34 μ L of isobutyl chloroformate were added in order, reacted from light with stirred at room temperature for 1h, the activated hapten solution for capsaicinoids was obtained.

The activated hapten solution for capsaicinoids was added dropwise into 10mL of 4.5mg/ml OVA solution to react, the reaction buffer was 0.2M pH8.0 phosphate buffer. The reaction was carried out under magnetically stirred at room temperature for 4 h. The reaction solution was placed in a dialysate bag, stirred and dialyzed in 0.01M pH7.4 PBS at 4 °C, changed dialysate solution every 4h, dialyzed for 72h in total. The artificial antigen-coating antigen for capsaicin was obtained. Ultraviolet-visible spectrum of continuous scanning spectra is shown in Figure 2, identification result shows that the artificial antigen is successfully coupled.

Example 4 Preparation of universal specific antibody for capsaicinoids

The prepared artificial complete antigen (immunological antigen) for capsaicinoids according to example 2 was used to immunize Japanese big ear rabbits of over 3kg. The average amount of antigen each time was about 0.54mg (based on the amount of the carrier protein). The immunological antigen which was emulsified in the same volume of Freund's incomplete adjuvant was used to immunize for the first time, the rabbits were injected by multiple sites subcutaneous injection; after the first immunization, the immunological antigen which was emulsified in the same volume of Freund's incomplete adjuvant was injected into the rabbits by multiple sites subcutaneous injection at their back at 3 weeks interval and following every 2 weeks interval. Start from the fourth time, one week after each immunization, the blood was collected from the rabbit auricular veins to be detected titer by non-

competitive indirect ELISA. After fifth immunization, the rabbit was collected blood from carotid artery thereof to death. The collected blood was placed at 37°C for about 1h, 4°C overnight, centrifuged, got the supernatant, a small amount of mixed volumetric glycerol was temporary reserved at -20°C to spare. The rest of antiserum was purified by caprylic acid-ammonium sulfate method to obtain freeze-drying antibody, reserved at -20°C to spare.

Example 5 Detection of universal specific antibody for capsaicinoids

1) The titer of universal specific antibody for capsaicinoids was detected by indirect non-competitive ELISA

Coating: The universal artificial complete antigen-coating antigen for capsaicinoids was diluted to 1µg/mL by 0.1mol/L pH9.6 carbonate buffer, 100 µL/well, reacted at 4°C overnight. The coating solution was drained out, washed full of well with PBST for 3 times, buckled to dry.

Blocking: 1% OVA PBST solution, 200 µL/well, incubated at 37°C for 1 h, washed full of well with PBST for 3 times, buckled to dry.

The specific immune response between antibody and antigen: universal specific antibody for capsaicinoids was diluted to 1mg/mL by 0.01M pH7.4 phosphate buffer. Then the antibody solution was diluted by multiple proportion dilution which start from 1:10,000, and added into coated well of different dilutions, the added amount was 100 µL/well, set the negative and blank control wells, the negative control serum was diluted for 2000 times, the blank one was added PBST only, incubated at 37°C for 1h, washed full of well with PBST for 3 times, buckled to dry.

Adding the secondary antibody: goat anti-mice second enzyme-labeled antibody was diluted for 5000 times by phosphate PBST solution, mixed, the added amount was 100 µL/well, incubated at 37°C for 1 h, washed full of well with PBST for 6 times, buckled to dry.

Coloration: chromogenic reagent (comprising 1 mg/mL tetramethylbenzidine 0.5mL, citric acid buffer 9.5mL, 1% urea hydrogen peroxide 32µL) was prepared when it will be used, 100 µL/well, incubated from light at 37°C for 15 min.

Termination: 2 mol/L H_2SO_4 , 50 μL /well, absorption value (ie OD₄₅₀ value) was immediately detected by the microplate reader at 450nm.

Reading result: when OD₄₅₀ value greater than or equal to 1, the highest dilution ratio of the antibody solution which OD₄₅₀ value corresponds to was the ELISA titer of the antibody, which was 128000.

2) The half maximal inhibitory concentration of universal specific antibody for capsaicinoids to capsaicin was detected by indirect competitive ELISA

(1) The working concentration of the antibody was determined by indirect ELISA described above, the antibody concentration which OD₄₅₀ value at about 1 corresponds to was the optimum working concentration.

(2) Coating, washing and blocking: operating the same way as indirect non-competitive ELISA.

(3) Preparing standard capsaicin solution: the capsaicin was formulated to 10 mg/ml mother solution by methanol solution. Before the sample added, the solution was diluted which start from 200 $\mu\text{g}/\text{mL}$ for 3 times by 10% methanol/PBS solution (0.01mol/L pH7.4), diluted for nine concentrations in total. 50 μL standard solution of capsaicinoids which was diluted by multiple proportion dilution was added to each well, and 50 μL antiserum with a dilution ratio of 160,000 was added to each well, reacted at 37 $^{\circ}\text{C}$ for 1 h.

(4) Adding the secondary antibody, coloration, termination and reading result: operating the same way as indirect non-competitive ELISA.

(5) Data processing: the standard curve was drawn with Logs of concentrations of capsaicin as abscissa and OD value which corresponds to concentrations of capsaicin as vertical axis, then the 50% inhibitory concentration was calculated based on the standard curve, which was 1.1 $\mu\text{g}/\text{mL}$.

3) The half maximal inhibitory concentration of universal specific antibody for capsaicinoids to dihydrocapsaicin was detected by indirect competitive ELISA

(1) The working concentration of the antibody was determined by the indirect ELISA described above, the antibody concentration which OD₄₅₀ value at about 1 corresponds to was the optimum working concentration.

- (2) Coating, washing and blocking: operating the same way as indirect non-competitive ELISA.
- (3) Preparing standard capsaicin solution: the dihydrocapsaicin was formulated to 10 mg/mL mother solution by methanol solution. Before the sample added, the solution was diluted which start from 200 $\mu\text{g/mL}$ for 3 times by 10% methanol/PBS solution (0.01mol/L pH7.4), diluted for nine concentrations in total. 50 μL standard solution of dihydrocapsaicin substances which was diluted as multiple proportion dilution was added to each well, then 50 μL antiserum with a dilution ratio of 160,000 was added to each well, reacted at 37 $^{\circ}\text{C}$ for 1 h.
- (4) Adding the secondary antibody, coloration, termination and reading result: operating the same way as indirect non-competitive ELISA.
- (5) Data processing: the standard curve was drawn with Log_{10} s of concentrations of dihydrocapsaicin as abscissa and OD value which corresponds to concentrations of dihydrocapsaicin as vertical axis, then the 50% inhibitory concentration was calculated based on the standard curve, which was 0.5 $\mu\text{g/mL}$.

4) The half maximal inhibitory concentration of universal specific antibody for capsaicinoids to synthetic capsaicin was detected by indirect competitive ELISA

- (1) The working concentration of the antibody was determined by the indirect ELISA described above, the antibody concentration which OD_{450} value at about 1 corresponds to was the optimum working concentration.
- (2) Coating, washing and blocking: operating the same way as indirect non-competitive ELISA.
- (3) Preparing standard capsaicin solution: the synthetic capsaicin was formulated to 10 mg/ml mother solution by methanol solution. Before sample added, the solution was diluted which start from 200 $\mu\text{g/mL}$ for 3 times by 10% methanol/PBS solution (0.01mol/L pH7.4), diluted for nine concentrations in total. 50 μL standard solution of synthetic capsaicin substances which was diluted by multiple proportion dilution was added to each well, then 50 μL antiserum with a dilution ratio of 160,000 was added to each well, reacted at 37 $^{\circ}\text{C}$ for 1 h.
- (4) Adding the secondary antibody, coloration, termination and reading result: operating the same way as indirect non-competitive ELISA.
- (5) Data processing: the standard curve was drawn with Logs of concentration of synthetic capsaicin as abscissa and OD value which corresponds to concentration of

synthetic capsaicin as vertical axis, then the 50% inhibitory concentration was calculated based on the standard curve, which was 1.8 μ g/mL.

5) The half maximal inhibitory concentration of universal specific antibody for capsaicinoids was detected by indirect competitive ELISA

(1) The working concentration of the antibody was determined by the indirect ELISA described above, the antibody concentration which OD₄₅₀ value at about 1 corresponds to is the optimum working concentration.

(2) Coating, washing and blocking: operating the same way as indirect non-competitive ELISA.

(3) Preparing standard capsaicin solution: the capsaicinoids was formulated to 10 mg/mL mother solution (which capsaicin, dihydrocapsaicin and synthetic capsaicin in ratio of 1: 1: 1) by methanol solution. Before the sample added, the solution was diluted which start from 200 μ g/mL for 3 times by 10% methanol/PBS solution (0.01mol/L pH7.4), diluted for nine concentrations in total. 50 μ L standard solution of capsaicinoids substances which was diluted by multiple proportion dilution was added to each well, then 50 μ L antiserum with a dilution ratio of 160,000 was added to each well, reacted at 37 $^{\circ}$ C for 1 h.

(4) Adding the secondary antibody, coloration, termination and reading result: operating the same way as indirect non-competitive ELISA.

(5) Data processing: the standard curve was drawn with Logs of concentration of capsaicinoids as abscissa and OD value which corresponds to concentration of capsaicinoids as vertical axis, then the 50% inhibitory concentration was calculated based on the standard curve, which was 0.7 μ g/mL.

In summary, universal artificial hapten and universal artificial complete antigen for capsaicinoids can be used to prepare high affinity, high sensitivity specific antibody for capsaicin.

Example 6

a Preparation of hybridoma cell line YQQD8

1. Animal immunization

Five BALB/c mice of the age of 7 weeks were purchased, and immunized with the above artificial antigen-immunological antigen for capsaicinoids. After

immunological antigen and the same volume of quick immunoadjuvant (QuickAntibody-Mouse5W) (Purchased from Beijing Biodragon Immunotechnologies Co., Ltd) were rapidly mixed for the first immunization, that injected into mice at calf muscle to immunize mice. Strengthen immunity in the same way at 21 day and 42 day respectively. The doses of immunological antigen for 3 immunizations were the same, which were 12.5 μ g for each mouse. Eight days after each immunization, took blood from the caudal vein of the mice, separated serum, the titer of the mice serum were detected by indirect ELISA. Eight days after third immunization, collected blood from the caudal vein of the mice, separated serum, the titer of the mice serum was detected by indirect ELISA, the sensitivity of mice serum was detected by indirect competitive ELISA, strengthen immunity of the mice for the last time which corresponds to serum with high affinity and high sensitivity, injected mice by intraperitoneal injection when strengthen immunity, the amount of immunological antigen was twice as much as before, without mixing with the adjuvants.

2. Cell fusion

Three days after the last immunization, 50% (by weight) of polyethylene glycol which was the PEG (molecular weight 1500) was used as the fusion agent, cell fusion was performed in conventional methods, the specific steps as follows: The immune mice was killed under aseptic condition, separated splenocyte and mixed with mice myeloma cells SP2/0 with a number ratio of about (5-10):1, mixed cells were washed by RPMI-1640 basal medium and were fused by 50% PEG for 1 min, then filled RPMI-1640 basal medium, centrifuged, removed the supernatant, fused cells which were fused by mice splenocyte and mice myeloma cells SP2/0 were resuspended by 72mL RPMI-1640 complete medium, the resuspended cells were added dropwise into a 96 well cell incubated plate, 2 drops/well, incubated at 37 $^{\circ}$ C in carbon dioxide incubator, wherein RPMI-1640 complete medium comprising 20% (volume percent) of fetal bovine serum, 10% (volume percent) of Clone easy[®] medium and 1% (weight percent) of hypoxanthine-aminopterin-thymidine(ie, HAT); Semisolid RPMI-1640 complete medium refers to RPMI-1640 complete medium with 1% (weight percent) of methylcellulose added. The above SP2/0 was purchased from Shanghai Fanke Biotechnology Co., Ltd.; Clone easy[®] medium was purchased from Beijing Biodragon Immunotechnologies Co., Ltd; RPMI-1640 basal medium was purchased

from Hyclone Corporation; 1% hypoxanthine-aminopterin-thymidine was purchased from Sigma-Aldrich Corporation.

3. Screening and Cloning of Cell Lines

Antibody detection was carried out when the cell colony on 96-well plate grew to reach 1/2 size of the well bottom, and medium turned to yellow. The wells with growing hybridoma cell were screened by ELISA, screening was performed in two steps, the positive wells with anti-capsaicin but without anti-carrier protein was screened by indirect ELISA in the first step; the positive wells which were screened from the first step were detected by indirect competitive ELISA in the second step, capsaicin was used as the competitive antigen, the competitive antigen and the antigen which was immobilized on an ELISA plate were competitively bound to antibody in the supernatant, wherein the antibody that was bound to capsaicin will be washed off in the subsequent step, the antibody that bound to the coated antigen was immobilized on the ELISA plate, would be colored after added HRP marked goat anti-mouse antibody and coloring solution. The higher concentration of the competitive antigen for capsaicin is, the shallower the color is, the lower the absorbance value at 450 nm. The wells were selected, which had higher absorbance value and higher sensitivity when the competitive antigen was not add (Here the higher absorbance value refers to the detected absorbance of the well which competitive antigen is zero, higher sensitivity refers to that competitive antigen concentration IC_{50} value was small when the inhibition rate was 50%). Semisolid medium was used to culture subclone cells, after cloning and then waiting cell colony grew to a size to be visible to naked eye, each cell colony was separately picked into liquid medium, using the same two-step screening method for detection, after repeating cloning once, the hybridoma cell line YQQD8 was finally obtained by screening. The cell line YQQD8 was deposited at China Center For Type Culture Collection (CCTCC) on April 23, 2015, wherein deposited address was Wuhan University, Wuhan, PR China, the preservation number was CCTCC NO. C201534, and named as the hybridoma cell line YQQD8.

b Detection of sequences of variable regions of universal monoclonal antibody hybridoma cell line YQQD8 antibody for anti-capsaicin, dihydrocapsaicin, synthetic capsaicin

(1) Extracting total RNA: The total RNA which can produce the hybridoma cell line YQQD8 was extracted by using total RNA extraction kit from TIANGEN Corporation and following the instruction;

(2) Synthesizing cDNA: The total RNA which was obtained in step 1 was used as template, oligo(dT)15 was used as primer, reverse transcription was performed according to the instruction of SuperScript™-2II reverse transcriptase, the first chain of cDNA was synthesized; primer oligo(dT)15 was purchased from Invitrogen;

(3) Cloning variable gene by PCR method: The primers were designed according to the conserved locus of mice antibody gene sequence in GENE BANK, light chain variable region gene and heavy chain variable region gene in the antibody were amplified with cDNA as a template. The PCR program was: 94℃ 30s, 55℃ 45s, 72℃ 1min, amplified for 30 cycles, finally extended at 72℃ for 10 min. The PCR products were electrophoretic separated by 1% (weight percent) agarose gel, DNA fragments were purified to recycle by using the kit, linked into carrier pMD18-T, Escherichia coli DH5α competent cell was transformed, picked positive clones, sent to Shanghai Sunny Biotechnology Co., Ltd. for sequencing. Wherein the sequences of the primers were: the primers of heavy chain variable region comprised: 5'-GAG GTG AWG STG GTG GAG TC-3, (20mer) 和 5'-GAG GAG ACG GTG ACT GAG GT-3, (20mer) , wherein S、R 和 W were degenerate bases, M=A/C , R=A/G, S=C/G, W=A/T, the primers of light chain variable region comprised: 5'-RTT KTG ATG ACC CAR AC -3, (17mer) 和 5'-ACG TTT KAT TTC CAG CTT GG -3, (20mer) .

The result of obtained gene sequence: Coding gene sequence length of heavy chain variable region was 313bp, the sequence was shown in SEQ ID NO:1, according to obtained gene sequence, it can be deduced that heavy chain variable region coded by the above gene sequence comprised 104 amino acids, the sequence was shown in SEQ ID NO:3. Coding gene sequence length of light chain variable region was 299bp, the sequence was shown in SEQ ID NO:2, according to obtained gene sequence, it can be deduced that light chain variable region coded by gene sequence comprised 99 amino acids, the sequence was shown in SEQ ID NO:4.

c Preparation, Purification, subtype and characteristic identification of universal monoclonal antibody for anti-capsaicin, dihydrocapsaicin, synthetic capsaicin

Universal monoclonal antibody hybridoma cell line YQQD8 for anti-capsaicin, dihydrocapsaicin, synthetic capsaicin which was obtained from example 2 was injected into BALB/c mice, which was pre-treated by incomplete Freund Adjuvant, the ascites of the mice was collected, the antibody was purified by caprylic acid-ammonium sulfate method, the specific operations comprised: the ascites of the mice was filtered by using the double filter paper, 4 μ m, 12000r/min centrifuged for 15min, drew supernatant, the obtained ascites supernatant were mixed with the 3 times volume of acetate buffer, the pH of the mixture was adjusted to 4.7 by using 2 mol/L hydrochloric acid solution, n-octanoic acid was added slowly under stirring, the volume of n-octanoic acid which was required per milliliter ascites was 33 μ L, mixed at room temperature for 30min, standing at 4 $^{\circ}$ C for 2h, 12000r/min centrifuged for 30min, removed precipitation, after the obtained supernatant was filtered by using the double filter paper, phosphate buffer with volume as 1/10 filtrate volume, 0.1mol/L of molar concentration and pH7.4 was added, the pH of the mixture was adjusted to 7.4 with 2 mol/L sodium hydroxide solution, pre-cooled at 4 $^{\circ}$ C, ammonium sulfate was slowly added to reach the final concentration of ammonium sulfate which was 0.277g/mL, standing at 4 $^{\circ}$ C for 2h, 12000r/min centrifuged for 30min, removed supernatant, the obtained precipitation was resuspended by using phosphate buffer with volume as 1/10 filtrate volume, 0.1mol/L of molar concentration, loaded into dialysate bag, 0.01 mol/L phosphate buffer was dialyzed, the well dialyzed protein solution was frozen at -70 $^{\circ}$ C in a refrigerator, then freeze-dried by using a freeze dryer, freeze-dried powder was collected, which were the purified universal monoclonal antibodies for anti-capsaicin, dihydrocapsaicin, synthetic capsaicin, the antibody was reserved in a refrigerator at -20 $^{\circ}$ C to spare; wherein the acetate buffer was obtained by 0.29g of sodium acetate, 0.141mL of acetic acid were added water to a 100mL volume; wherein the 0.1mol/L phosphate buffer were obtained by 0.8g sodium chloride, 0.29g of disodium hydrogen phosphate dodecahydrate, 0.02g of potassium chloride and 0.02g of potassium dihydrogen phosphate added water to a 100mL volume.

The subtype of universal monoclonal antibody for anti-capsaicin, dihydrocapsaicin, synthetic capsaicin which was secreted by hybridoma cell line YQQD8 was identified as IgG₁ by using commercially available subtype identification kit.

The titer of YQD8 mice ascites antibody which was detected by conventional non-competitive ELISA could reach 2.56×10^6 , that is, the solution which mouse ascites antibody solution was diluted for 2.56×10^6 time was detected as positive. Detected by conventional indirect competitive ELISA, the half maximal inhibitory concentrations IC_{50} of the antibody for capsaicin, dihydrocapsaicin, synthetic capsaicin were 8.5ng/mL, 5.0ng/mL, 13.5ng/mL in order, and cross reactivity range of capsaicin, dihydrocapsaicin, synthetic capsaicin was 62.9%-170%.

Nucleotide and amino acid sequence listing

<110> The Oil Crops Research Institute (OCRI) of the Chinese Academy of Agricultural Sciences (CAAS)

<120> Universal artificial hapten and universal artificial complete antigen for capsaicinoids and application thereof

<160> 4

<170> PatentIn version 3.3

<210> 1

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<212> DNA

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<213>mice

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Ser Tyr Val Gly Thr Asn Lys Tyr Asn Pro Ser Leu Lys Asn Arg 60
lie Ser lie Thr Arg Asp Thr Ser Lys Asn Gin Phe Phe Leu Lys 75
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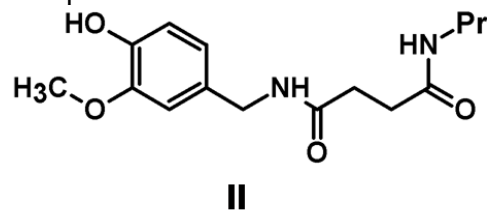
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Ile Tyr Leu Val Ser Lys Leu Asp Ser Gly Val Pro Asp Arg Phe	60
Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg	75
Val Glu Ala Glu Asp Leu Gly Ile Tyr Tyr Cys Val Gln Gly Thr	90
His Phe Pro Trp Thr Phe Gly Gly Gly	99

CLAIMS

1. A universal artificial complete antigen compound for capsaicinoids, wherein the compound has the molecular structure as shown in formula II:



, Pr is carrier protein.

2. The universal artificial complete antigen compound for capsaicinoids according to claim 1, wherein the carrier protein is bovine serum albumin BSA, ovalbumin OVA or keyhole limpet hemocyanin KLH.

3. The process for synthesizing the universal artificial complete antigen compound for capsaicinoids according to claim 1, wherein the universal artificial hapten for capsaicinoids is activated by N,N'-dicyclohexylcarbodiimide DCC and N-hydroxysuccinimide NHS, and then coupled with the carrier protein to obtain the universal artificial complete antigen compound.

4. The process for synthesizing the universal artificial complete antigen compound for capsaicinoids according to claim 3, wherein comprising the specific steps below:

(1) Activation of universal artificial hapten for capsaicinoids: universal artificial hapten for capsaicinoids is reacted with N-hydroxysuccinimide (NHS) at room temperature for 30min-2h in N,N-dimethylformamide, then dicyclohexylcarbodiimide in N,N-dimethylformamide solution is added, reacted at room temperature for 4-6h, standing overnight at 4°C, got supernatant, that is the activated artificial hapten for capsaicinoids, wherein the molar ratios of N-hydroxysuccinimide (NHS) to dicyclohexylcarbodiimide are 1:(1- 1.2);

or artificial hapten for capsaicinoids is dissolved in N,N-dimethylformamide, after tributylamin and isobutyl chloroformate are added sequentially, reacted at room temperature for 0.5-3h, got reaction solution, that is the activated artificial hapten for capsaicinoids, wherein the molar ratios of tributylamin to isobutyl chloroformate are 1:(1-1.2);

(2) The supernatant obtained in the above step (1) which is the activated universal artificial hapten for capsaicinoids is added dropwise into the phosphate buffer with carrier protein, wherein carrier protein has the concentration greater than or equal to 2mg/mL, reacted at room temperature for 4-8h, whereby: wherein the molar ratio of activated universal artificial hapten for capsaicinoids to carrier protein is great than 10:1, then dialyzed to obtain universal artificial complete antigen for capsaicinoids.

5. The process for synthesizing the universal artificial complete antigen compound for capsaicinoids according to claim 3, wherein the phosphate buffer with carrier protein was obtained by dissolving the carrier protein in 0.2M pH8.0 phosphate buffer solution.

6. The use of universal artificial complete antigen compound for capsaicinoids according to claim 1 in the preparation of specific antibody for capsaicinoids.

7. The use according to claim 6, wherein the steps of the use comprises: the Japanese big ear rabbits are immunized with the above universal artificial complete antigen for capsaicinoids, after immunized for 4-6 times, blood is collected from rabbit carotid artery thereof to the rabbit's death, after the collected blood is centrifuged, antiserum is purified by caprylic acid-ammonium sulfate method to obtain universal antibody for capsaicinoids, which is the globulin that can have specific immune response with capsaicinoids.