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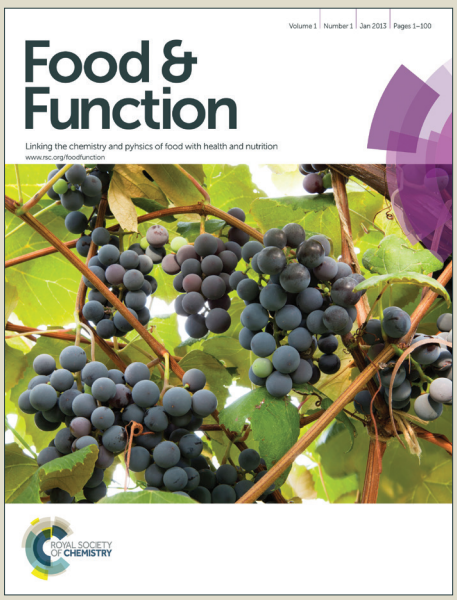

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Immunomodulating effects of casein-derived peptide QEPVL and QEPV on lymphocytes in vitro and in vivo

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Abstract

Lymphocytes serve an important function in mediating specific immune responses. When the body is stimulated by internal or external antigens, activated lymphocytes proliferate to clear pathogens by secreting antibodies or cytokines. Some bioactive peptides were isolated from fermented milk in previous studies. One of the peptides, Gln-Glu-Pro-Val-Leu (QEPVL), was synthesized and used in this experiment. Results show that QEPVL can significantly activate lymphocytes both in vitro and in vivo. QEPVL can also increase the lymphocyte proliferation rate and cyclic AMP levels. This positive regulation had a dose-effect relationship within certain concentration ranges. QEPVL can also inhibit LPS-induced inflammation by regulating nitric oxide release and the production of the cytokines IL-4, IL-10, IFN-γ, and TNF-α in vivo. Digesting QEPVL in artificial gastrointestinal juice yields the digestion product Gln-Glu-Pro-Val (QEPV), which exhibits bioactivities similar to those of QEPVL in vitro. Overall, QEPVL has significant immunomodulating effects on lymphocytes and contributes to inflammation treatment through the oral route as a functional food ingredient.

Introduction

Bioactive peptides are specific protein fragments that positively affect body functions and may ultimately influence health. Immunomodulating peptides comprise the second type of bioactive peptides derived from milk after opioid peptides and have been proven to exert biological effects.[1] Mercier et al. found that some whey protein isolates, their enzymatic digestions, and peptide fractions stimulated the proliferation of murine-resting splenocytes in vitro and suggested that some immunomodulating peptides can be released by enzymatic digestion.[2]

T and B Lymphocytes are known to serve a crucial function in immune responses. When the body is attacked by pathogens, antigens bind to B lymphocyte cell receptors. This interaction activates B lymphocytes and stimulates them to produce antibodies, including IgM, IgG, IgA, and IgE.[3, 4] Similar to B lymphocytes, the activation of T lymphocytes also requires costimulatory signals and the binding of the T lymphocyte cell receptors to the antigen. Mature T lymphocyte cells are divided into two subgroups depending on the CD molecules they possess: $CD4^+$ T cells and $CD8^+$ T cells.[5] $CD4^+$ T cells are called helper T lymphocytes (Th), which serve an immune regulatory function by secreting cytokines to enhance immune function.[6] $CD8^+$ T cells, called cytotoxic T lymphocytes (CTL), are mainly immunosuppressive and function by killing target cells.[7] Cyclic AMP (cAMP), known as an intracellular second messenger, has been proven to interact with Th cells through cytokine secretion and expression of surface molecules.[8] After being activated and proliferated by external antigens, cytokines are secreted by Th cells and can be classified into Th1 and Th2. Th1 and Th2 serve different functions in immune response.[6] IFN-γ, IL-2, and TNF-α are mainly secreted by Th1 to promote cellular immunologic response, whereas IL-4, IL-10, and IL-13 are mainly secreted by Th2 to promote humoral immunoresponse.[9] Previous studies indicate that IFN-γ has antitumor and antiviral bioactivities, as well as enhance CTL and macrophage cell viability. By contrast, IL-4 is known to induce the growth and differentiation of Th2.[10, 11] These two types of cytokines were found to have an antagonistic effect on each other, as reflected in the equilibrium relationship of Th1/Th2, which was considered as a Th cell cytokine expression mode existing in the body.[9, 12, 13] In medical research, the ratio of IFN-γ and IL-10 was also used to evaluate the balance of Th1/Th2 to be more completely designed.[14]

Inflammation is a protective response of the human body by which pathogens are produced

through the production of various pro-inflammation mediators, such as IFN-γ, TNF-α, IL-6, and nitric oxide (NO). However, the overproduction of such pro-inflammatory factors by immune cells damages body tissues and decreases the immune function.[15] Th2 positively affects inflammation by secreting anti-inflammatory IL-4 and IL-10, thus inhibiting the overproduction of pro-inflammation factors. If inflammation becomes fatal, Th1/Th2 then becomes unbalanced. With the large production of IFN-γ and TNF-α, Th1 cells are activated while Th2 cell viability is usually decreased.[12–14]

In our previous work, we isolated some peptides from fermented milk produced by *Lactobacillus helveticus* and found that one peptide significantly affected lymphocyte proliferation in vitro.[16] This peptide fragment was identified as Gln-Glu-Pro-Val-Leu (QEPVL). In this study, QEPVL was digested in artificial gastrointestinal juice to study the bioactivity of the digestion product and was demonstrated to be resistant to digestion because the digested product showed the same bioactivity in vitro. We also studied the regulating effects of QEPVL for lymphocyte proliferation, activity, and secretion of cytokine both in vitro and in vivo.

Materials and methods

2.1 Peptides

Synthetic QEPVL peptides were purchased from China Peptide Co., Ltd.

2.2 Gastrointestinal digestion

Experimental methods of gastrointestinal digestion were performed according to Alting et al. and Hernández–Ledesma et al.[17, 18] The peptide solution was dissolved in neutral ultrapure water at a concentration of 500 μg/mL. Pepsin (Sigma, China) was then added at an amount of 20 mg per gram of peptide. Hydrochloric acid was mixed into the solution until the pH value reached 2.0. The solution was heated in a water bath at 37 °C for 90 min. NaOH solution (1 M) was added until the pH value reached 7.5. Corolase PP (AB, Germany) was added at 40 mg per gram of peptide, heated in water bath at 37 °C for 150 min, and then quickly heated in a water bath at 95 °C for 5 min to stop the enzyme activity. The sample was freeze-dried and stored at -20 °C for further analysis. In this experiment, Corolase PP was selected instead of single trypsin because Corolase PP is a mixture of multiple digestive enzymes, and the digestive environment in the body can be better simulated by using an enzyme mixture than by using a single trypsin or any other enzyme.

2.3 UPLC-Q-TOF-MS analysis

The lyophilized powder was dissolved in 50 μL of water and 450 μL of absolute ethyl alcohol, then stored in a -20 °C refrigerator for 20 min. The mixture was then centrifuged at 1500 g for 30 min to collect 400 μL of the supernatant for ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) analysis. TIC-UPLC-Q-TOF facilitated the total ion chromatography to separate each component of peptides after digestion. UPLC-Q-TOF-MS was then used to analyze the amino acid sequences of each peptide in the mixture.[19] Results obtained were analyzed using MarkerLynx v4.0.

2.3.1 UPLC

Chromatographic separation was performed on a Hypersil GOLD C_{18} column (100 mm × 2.1 mm, 1.9 μm) at a column temperature of 45 °C. The mobile phase consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile, v/v). The optimized UPLC elution condition was set at 99% to 50% A. The flow rate was set at 0.4 mL/min. The injection volume was 5 μL.

2.3.2 Q-TOF-MS

The scan range was from 80 m/z to 1000 m/z, whereas the scan time was set to 0.3 s. Mass spectrometry was operated using electrospray ionization for positive electrospray modes. The capillary and cone voltages were set as 3.0 kV and 35 V, respectively. The desolvation gas was set to 500 L/h at a temperature of 300 °C. Leucine–enkephalin at a concentration of 200 ng/mL was used for the lock mass to ensure accuracy.

2.4 Isolation of mice lymphocytes

Six-week-old male Balb/c mice (SLAC, China) received food and water ad libitum during one week of acclimation, after which the spleens were collected during week 2 under sterile conditions. All animal work was performed in compliance with the protocol approved by the Institutional Animal Care and Use Committee at Shanghai Jiao Tong University [Animals Use License No. SYXK (HU) 2013-0050] and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23). The spleen was abraded gently and rinsed with RPMI1640 medium (GIBCO, USA) repeatedly and lightly to obtain the cell suspension. Lymphocyte separation medium (Solarbio, China) was added and centrifuged at 2000 g for 20 min at 4°C. Thereafter, three layers can be clearly observed in the tubes. The top layer turned out to be the medium, whereas the bottom part was cell debris. Lymphocyte crude extracts were obtained from the liquid located in the middle layer. Crude extracts were mixed with isopyknic RPMI 1640 medium, after which lymphocytes were pelleted by centrifugation at 1500 g for 10 min at 4 °C. RPMI 1640 medium was then added to obtain the lymphocyte suspension.[20]

2.5 Cell proliferation assay

A 100 μL single cell suspension was added into each well in 96-well plates at a concentration of 1×10^5 cells/well.[21] Cell viability was determined by a CCK-8 assay (KeyGEN, China). Lymphocyte proliferation rates were detected with or without stimulation of mitogen Con A (Sigma, USA). Both peptides and mitogen were dissolved in RPMI 1640 medium, thus avoiding any other effect on cell growth.

In the experiment without the stimulation of Con A, lymphocytes were treated with QEPVL solution at different final concentrations ranging from 50 μg/mL to 1500 μg/mL for 48 h or treated with Con A at final concentration of 1 μg/mL. The negative groups were treated with RPMI 1640 medium at the same volume for 48 h. In another experiment, all lymphocytes were stimulated with mitogen at a final concentration of 1 μg/mL. The QEPVL solution was immediately added to the test groups at different final concentrations ranging from 1 μg/mL to 1000 μg/mL for 48 h. The mouse lymphocyte proliferation rate was determined using the CCK-8 method according to manufacturer's instructions. CCK-8 was also added into eight wells that only contain RPMI 1640 medium as the blank wells to avoid the influence of medium color. The final volumes of these wells remained the same as those in other wells. The stimulation index (SI) was calculated using the following equation:

SI= (absorbance of peptide-treated cells - absorbance of blank wells) / (absorbance of negative control cells - absorbance of blank wells).

The QEPV effect on lymphocyte proliferation was also tested at different final concentrations, with or without the stimulation of Con A. The experimental protocols were as above described.

2.6 cAMP measurement

Lymphocytes were seeded in 96-well plates at a concentration of 5×10^5 cells/well. QEPVL or QEPV was added to the experimental group, and final concentrations were divided into three

groups: 10, 100, and 1000 μg/mL. RPMI 1640 medium was also added to the control group at the same volume. Cells were cultured for 36 h, and then 100 μL of 0.1 M HCl was added to each well to lyse cells for 10 min. cAMP levels in the supernatant were determined by ELISA using an ELISA kit (NewEast Biosciences, USA) according to the manufacturer's instructions. All samples and standards were measured thrice.

2.7 Animal groups and inflammation induction

Ninety five-week-old male Balb/c mice were randomly separated into three groups (n = 30). The control and LPS groups both received PBS, whereas the QEPVL plus LPS group received QEPVL at 200 mg/kg, all by oral administration for three weeks. The LPS and QEPVL plus LPS groups were i.p.-injected with LPS (Sigma, USA) from *Escherichia coli 0111: B4* (5 mg/kg), whereas the control group was injected with PBS. The 5 mg/Kg LPS dose was applied according to O'Bryan et al., thus causing inflammation and avoiding endotoxic shock simultaneously. The potential of QEPVL as a functional food ingredient to help regulate inflammation, not as a drug type to cure inflammation, was tested.[22] At 1, 2, 4, 6, and 12 h after i.p. injection, blood was collected from six mice in each group.

2.9 Measurement of NO

Mice serum was stored at 4 °C for NO analysis. The NO level was measured using a total NO kit (Beyotime, China) according to manufacturer's instructions. All samples were measured twice.

2.10 Measurement of cytokine by flow cytometry

Mice serum was stored at -20 °C for cytokine analysis. The cytokine level (IL-4, IL-10, IFN-γ, and TNF-α) was measured by a cytometric bead array kit (BD, USA) according to the manufacturer's instructions. The data acquired were analyzed using the FCAP Array Software. All samples were measured twice.

2.11 Statistical analysis

All data were expressed as mean ± standard deviations for three independent experiments performed with SPSS19.0 software. Experiment data were analyzed by one-way analysis of variance (ANOVA) and Duncan's new multiple range test. Differences were considered to be significant if $p < 0.05$.

Results

3.1 Effects of gastrointestinal digestion

As shown in the total ion chromatograms in Fig. 1, the peptides remained the same after pepsin digestion. However, new peaks appeared after Corolase PP digestion and became the main peaks, indicating that QEPVL was digested. The mass-spectrograms of all peaks under the three digestion conditions were observed to calculate the molecular weight of the peptide fragment. Fig. 2-1 shows peak b1 with a corresponding molecular weight of 568.2966 Da, which is consistent with the molecular weight of QEPVL without a water molecule. Fig. 2-2 shows peak b2 with a corresponding molecular weight of 585.3223 Da, which is the actual molecular weight of QEPVL. Fig. 2-3 shows peak b4 with a corresponding molecular weight of 472.2409 Da, whereas peak b3 is shown in Fig. 2-4 with a molecular weight 455.2092 Da, which is similar to peak b4 without a water molecule.

All the possible fracture mechanisms of QEPVL are listed in Table 1. Based on all the possible fracture mechanisms and the molecular weight of water, peak b4 was determined to be QEPV (gastrointestinal digestion product of QEPVL) and corresponds to its fragment size. The molecular

weight of 472.2409 Da equates to QEPVL reducing L. The peak areas in Fig. 1 were calculated to obtain Table 2 and determine digestibility. As shown in Table 2, the QEPVL acreage percentage decreased from 100% to 13.74%, indicating that 86.26% of the peptide was decomposed.

3.2 Effects of QEPVL and QEPV on lymphocyte proliferation

In this experiment, CCK-8 assays were used to determine the QEPVL and QEPV effects on lymphocyte proliferation.

The QEPVL, QEPV, and Con A effects on the proliferation were compared based on SI values, and the results are shown in Table 3. Con A at a concentration of 1 μg/mL could evidently improve the resting of lymphocyte proliferation. QEPVL significantly improved lymphocyte proliferation at different concentration levels from 50 μg/mL to 1500 μg/mL when resting lymphocytes in RPMI 1640 were regarded as the control group. However, QEPVL at 50 μg/mL and 1500 μg/mL showed the same regulatory effect on the proliferation rate, and SI reached the maximum when QEPVL was 300 μg/mL, indicating that 300 μg/mL could be the optimum concentration. As a degraded product of QEPVL, QEPV showed the same regulatory effect on resting murine splenocytes.

Based on the SI values calculated in Table 4, QEPVL significantly promoted lymphocyte proliferation obtained with 1 μg/mL Con A at concentrations ranging from 1 μg/mL to 1000 μg/mL ($p < 0.05$, $p < 0.01$). The maximum SI value was obtained when QEPVL was 300 μg/mL, indicating that this value could be the optimum concentration, which had the same tendency as shown before. As a degraded product of QEPVL, QEPV was also capable of significantly increasing lymphocyte proliferation at a concentration of 300 μg/mL ($p < 0.05$, $p < 0.01$).

3.3 Effects of QEPVL and QEPV on cAMP production

Unlike the control group, cAMP production was significantly increased by QEPVL at the concentration range of 10 μg/mL to 1 mg/mL ($p < 0.05$, $p < 0.01$), as well as by QEPV at all concentrations ($p < 0.01$) (Table 5). The dose-effect relationship also appeared in both peptides.

3.4 Effects of QEPVL on NO release in vivo

As shown in Fig. 3, unlike the control group at each time point, the NO release evidently increased in mice serum after LPS treatment for 4 h compared with the LPS and QEPVL plus LPS groups ($p < 0.05$). Although the increasing trend of NO release did not change during the inflammation process, the volume of NO release can be observably suppressed by the QEPVL pre-treatment unlike LPS group at the same time point ($p < 0.05$ and $p < 0.01$, respectively). The results reveal that NO release is lowered when the animal body has previously taken QEPVL and then stimulated by an inflammatory factor.

3.5 Effects of QEPVL on the production of cytokines in vivo

As shown in the preliminary experiments, the time-production tendencies of different cytokines were varied for the inflammatory mice. Thus, all blood samples were collected at 1, 2, 4, 6, 12 h after LPS treatment to observe the variation tendencies of cytokine production and the relationship with time in a more reasonable period. The results show that pro-inflammatory cytokines, TNF-α, and IFN-γ remarkably increased by LPS stimulation in the LPS group unlike the control group. The cytokine concentration reached the maximum at 1 h (Fig. 4-1) and 6 h (Fig. 4-2), respectively. In the QEPVL plus LPS group, pre-treated with QEPVL and the same LPS treatment, the variation tendencies of pro-inflammatory cytokines were observed to be similar to those of the inflammation group, but the cytokine concentrations were lower than those of the inflammation group at the same time point. This condition indicates that the production of pro-inflammatory

cytokines was inhibited although inflammation still existed. Changes in the anti-inflammatory cytokines IL-4 and IL-10 supported the hypothesis above. The production of IL-4, which was not detected in the inflammatory and healthy mice but increased to 4.35 pg/mL in the QEPVL plus LPS group, shows that Th2 was activated by QEPVL to contribute to anti-inflammation (Fig. 4-3). An increase in the serum IL-10 levels by pre-treatment of QEPVL was also observed unlike the other two experimental groups (Fig. 4-4). Results prove that Th1 cells were inhibited from producing pro-inflammatory cytokines unlike the LPS group, whereas Th2 cells were activated to serve an anti-inflammatory function. Thus, Th1/Th2 was decreased by QEPVL during the inflammation process.

Discussion

Immunomodulating peptides can function only when absorbed into the body and employed to act on the target organs as drugs or functional food. In the gastrointestinal tract, peptide function is easily affected by pH or various digestive enzymes, such as pepsin and trypsogen, thus becoming insufficient for the purpose of its origin.[23, 24] In this study, pepsin, Corolase PP, HCl, and NaOH were used to simulate digestion in the gastrointestinal tract. Corolase PP is a mixture of multiple digestive enzymes that mainly includes trypsin, zonolysin, aminopeptidase, and carboxypeptidase. Small amounts of pancrelipase and amylopsin were also included. The digestive environment in the body can be better simulated when using an enzyme mixture than using single trypsin or any other enzyme.[25] As shown in Fig. 1, QEPVL was slightly affected by acid and pepsin, but 86.26% of QEPVL was enzymatically degraded by Corolase PP into QEPV, which shows that QEPVL can be enzymatically degraded into QEPV in the intestinal tract and similarly functions as the main digestion product of QEPVL.

As the most fundamental factor, lymphocyte proliferation indicates whether it is beneficial or cytotoxic to lymphocytes. As shown in Table 3, both QEPVL and QEPV have a significant effect on lymphocyte proliferation at the same optimum concentration of 300 μg/mL. Given that they originate from fermented milk, they are much safer bioactive products and are not antigens for animal body.

The regulation of cAMP levels is a critical process for all type of cells that act as a second messenger to active immune cells. Clinical evidences suggest that cAMP and CD28 has beneficial effects on immune regulation by increasing the expression of IL-10, thus influencing Th1/Th2 balance.[26] The positive effect of QEPV on cAMP levels became stronger than QEPVL, which indicates that QEPV can activate more Th2 cells than QEPVL. Thus, QEPVL has a potential anti-inflammatory function in animal bodies when orally administrated to become QEPV in the animal intestinal tract.

T and B lymphocyte cells serve major functions in mediating specific immune responses. B lymphocytes mediate humoral immunity by producing immunoglobulins.[27] T cells have two main functions. CTL kills target cells such as virus-infected cells and tumor cells, whereas regulatory T cells regulate immune cells through positive or negative feedback mechanisms to maintain internal balance; the ratio of CD4 and CD8 indicates the balance between Th cells and cytotoxic T cells.[7, 28] Increased production of cytokines IFN-γ and TNF-α are usually associated with active inflammatory diseases, which is lethal to the body. IL-4 and IL-10 have been recognized as major anti-inflammatory cytokines suppressing pathogen clearance and infection-associated immunopathology.[14, 29] In the present study, IFN-γ/IL-4 and IFN-γ/IL-10 were selected to evaluate

the QEPVL function. The results show that when lymphocytes are attacked by LPS, QEPVL could significantly regulate Th1/Th2 cytokines by increasing IL-4 and IL-10 and decreasing IFN-γ production, which may activate Th2 to exert its immune responses. Whether QEPVL can activate Th2 cells should be further studied.

In conclusion, QEPVL can significantly activate lymphocytes and promote lymphocyte proliferation both in vitro and in vivo. QEPVL also has a positive regulation to inflammation by increasing cAMP levels, NO release, and cytokines production. The physiological activity of its digestion product QEPV shows the same regulatory effect in vivo. Thus, these findings suggest that QEPVL has significant immunomodulating effects and potential functions for inhibiting the inflammatory response as a bioactive peptide when previously administered.

Acknowledgement

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Table 1 The analysis of possible amino acid sequences from gastrointestinal digestion

Amino acid sequence	Molecular weight	Control group	Pepsin digestion	Corolase PP digestion
QEPVL	584.3170	+	+	+
QEPV	471.2329	-	-	+
L	113.0841	-	-	-
QEP	372.1645	-	-	-
VL	230.1630	+	+	+
QE	275.1117	-	-	+
PVL	327.2158	+	+	+
Q	128.0586	-	-	-
EPVL	456.2584	+	+	+

Table 2 The retention time and peak areas of gastrointestinal digestion

Sample	Retention time	Peak height	Peak area	Acreage percentage (%)
Control group	6.49	55491	3222.66	61.11
Control group	7.01	26030	2050.83	38.89
Pepsin digestion group	6.50	47494	2638.98	57.95
Pepsin digestion group	7.01	23604	1915.09	42.05
Corolase PP digestion group	3.46	3118	680.18	26.78
Corolase PP digestion group	5.36	12730	1511.09	59.49
Corolase PP digestion group	6.50	672	36.26	1.43
Corolase PP digestion group	7.10	6050	312.60	12.31

Table 3 Effects of QEPVL, QEPV and Con A on resting lymphocyte

Group	Concentration (μg/mL)	SI
Control	**-**	1Aa
QEPVL	**50**	1.198±0.039Ab
QEPVL	**100**	1.382±0.037Ab
QEPVL	**300**	1.587±0.030Bc
QEPVL	**500**	1.518±0.043Bc
QEPVL	**1000**	1.519±0.032Bc
QEPVL	**1500**	1.229±0.037Ab
QEPV	**100**	1.326±0.128Ab
QEPV	**300**	1.556±0.043Bc
QEPV	**1000**	1.473±0.053Bc
ConA	**1**	1.568±0.028Bc

Data shown are mean ± SD of the results of 8 cultures. Values within same row not sharing a common letter are significantly different. Capital and small letter indicate $p < 0.01$ and $p < 0.05$ respectively.

Table 4 Effects of QEPVL and QEPV on lymphocyte proliferation (with Con A stimulation)

Group	Concentration (μg/mL)	SI
Control	**0**	1Aa
QEPVL	**1**	1.156±0.099Ab
QEPVL	**10**	1.154±0.110Ab
QEPVL	**50**	1.241±0.044Ab
QEPVL	**100**	1.412±0.177Bc
QEPVL	**300**	1.472±0.099Bc
QEPVL	**500**	1.319±0.032Ab
QEPVL	**1000**	1.313±0.071Ab
QEPV	**50**	1.153±0.039Ab
QEPV	**100**	1.367±0.043Ab
QEPV	**300**	1.413±0.032Bc
QEPV	**500**	1.360±0.115Ab
QEPV	**1000**	1.248±0.353Ab

Data shown are mean ± SD of the results of 8 cultures. Values within same row not sharing a common letter are significantly different. Capital and small letter indicate $p < 0.01$ and $p < 0.05$ respectively.

Table 5 Effects of QEPVL and QEPV on cAMP production

Group	Concentration (μg/mL)	Levels of cAMP (pmol/L)
Control	**-**	0.067±0.126Aa
QEPVL	**10**	0.677±0.097Ab
QEPVL	**100**	1.794±0.017Bc
QEPVL	**1000**	2.042±0.654Bc
QEPV	**10**	1.283±0.355Bc
QEPV	**100**	3.900±0.355Bc
QEPV	**1000**	4.260±0.651Bc

Data shown are mean ± SD of the results of 8 cultures. Values within same row not sharing a common letter are significantly different. Capital and small letter indicate $p < 0.01$ and $p < 0.05$ respectively.

Figure Legends

Fig. 1. TIC-UPLC-Q-TOF analysis results of samples through gastrointestinal digestion. Peak b1: QEPVL without a water molecule; b2: QEPVL; b3: QEPV; b4: QEPV without a water molecule.

Fig. 2. UPLC-Q-TOF-MS analysis results of peaks b1, b2, b3, and b4 after the TIC-UPLC-Q-TOF analysis. Figure 2-1: Peak b1: QEPVL without a water molecule; Figure 2-2: b2: QEPVL; Figure2-4: b3: QEPV; Figure 2-3: b4: QEPV without a water molecule.

Fig. 3. Effects of QEPVL on NO release in three different groups after the injection times of 1, 2, 4, 6, and 12 hours. LPS: mice were orally administrated with PBS for 3 weeks and then injected with LPS at 5 mg/kg; QEPVL+LPS: mice were orally administrated with QEPVL at 200 mg/kg for 3 weeks and then injected with LPS at 5 mg/kg; Control: mice were orally administrated with PBS for 3 weeks and then injected with PBS. *: $p < 0.05$ (QEPVL+LPS group versus the LPS group). (The whole experiment was repeated thrice in parallel).

Fig. 4. Effects of QEPVL on cytokine expression in three different groups after the injection times of 1, 2, 4, 6, and 12 hours. LPS: mice were orally administrated with PBS for 3 weeks and then injected with LPS at 5 mg/kg; QEPVL + LPS: mice were orally administrated with QEPVL at 200 mg/kg for 3 weeks and then injected with LPS at 5 mg/kg; Control: mice were orally administrated with PBS for 3 weeks and then injected with PBS. Fig. 4-1: effects of QEPVL on TNF-α production; Fig. 4-2: effects of QEPVL on IFN-γ production; Fig. 4-3: effects of QEPVL on IL-4 production; Fig. 4-4: effects of QEPVL on IL-10 production. *: $p < 0.05$ (QEPVL+LPS group versus the LPS group). (The whole experiment was repeated thrice in parallel).

Fig. 1 TIC-UPLC-Q-TOF analysis results of samples

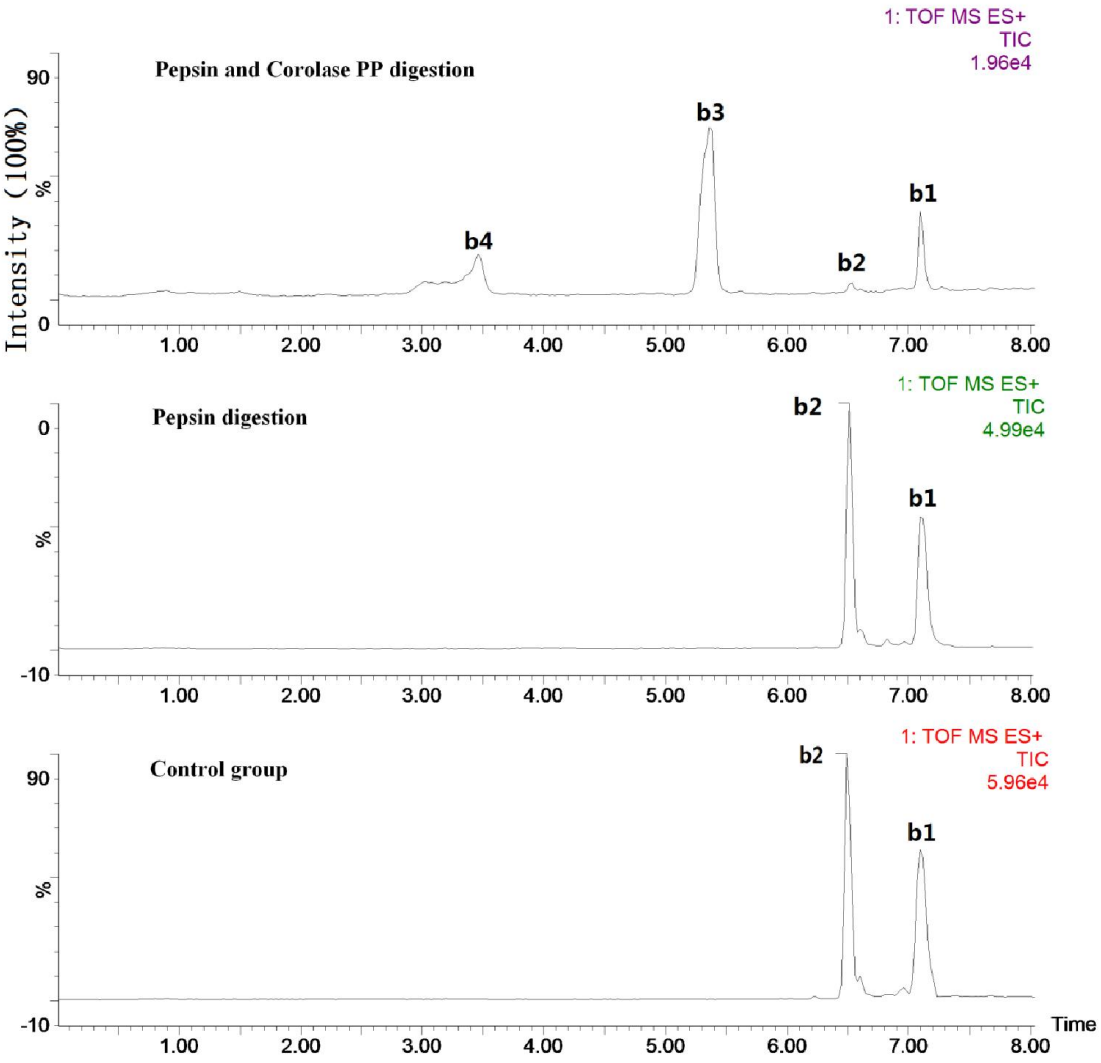


Fig. 2-1 UPLC-Q-TOF-MS spectrum of peak b1 from TIC-UPLC-Q-TOF analysis result

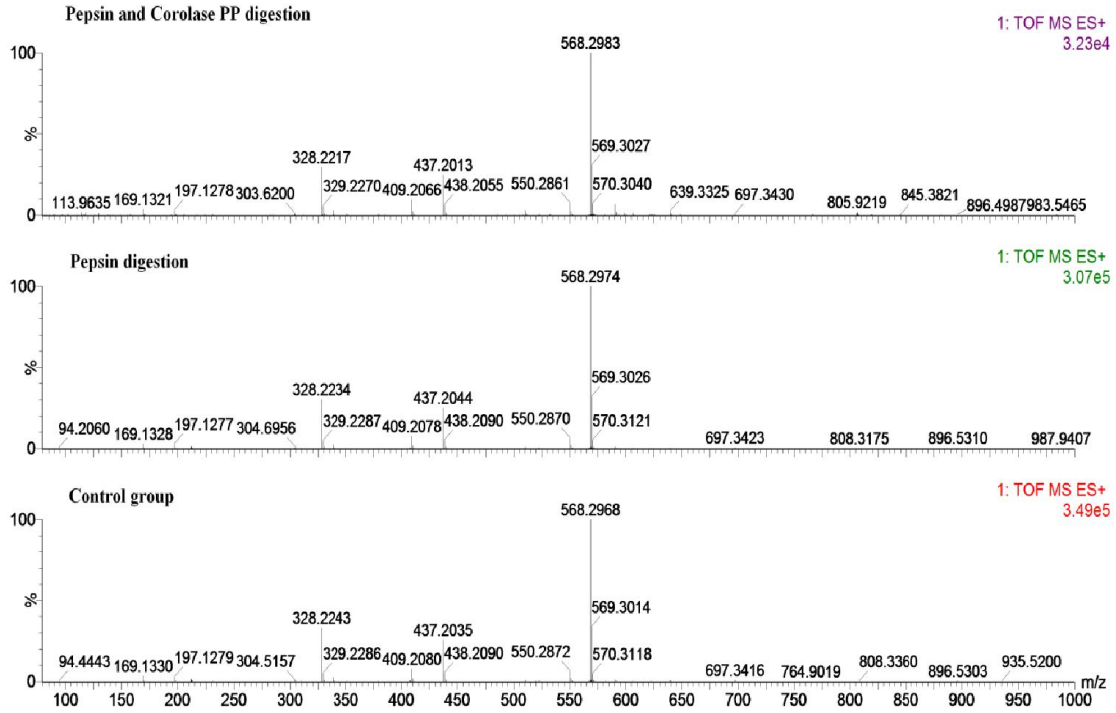


Fig. 2-2 UPLC-Q-TOF-MS spectrum of peaks b2 from TIC-UPLC-Q-TOF analysis result

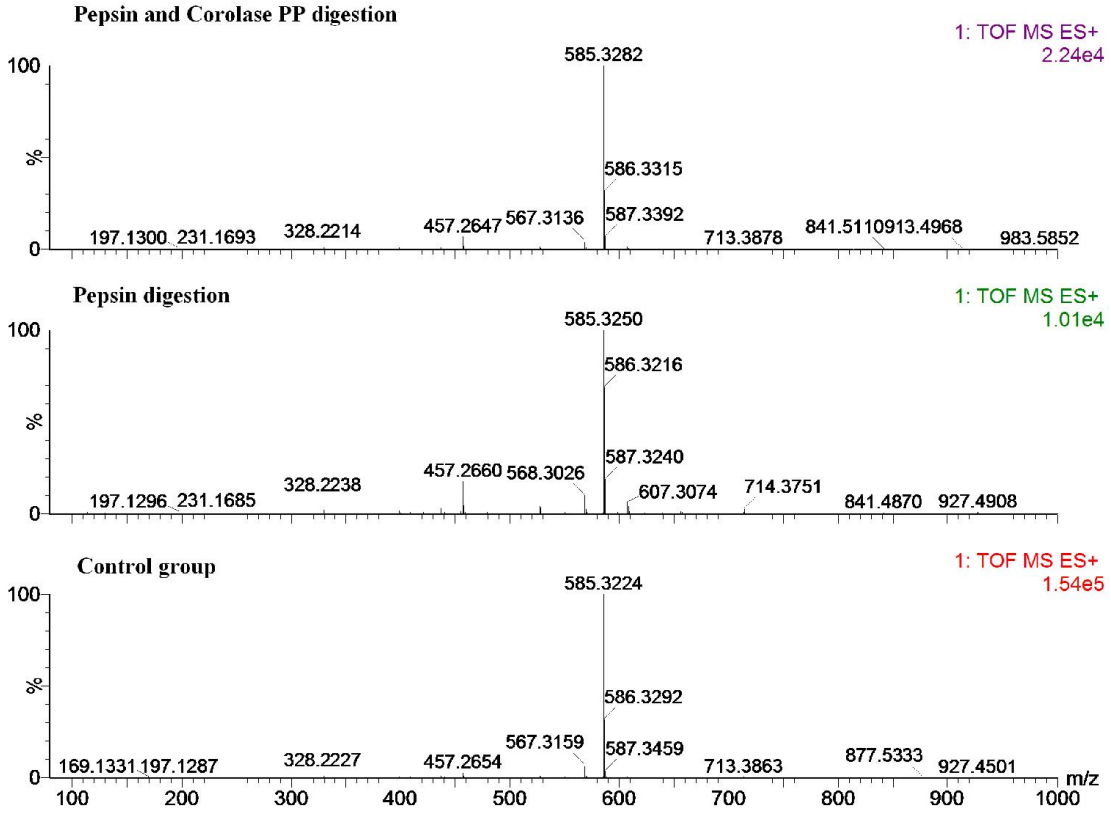


Fig. 2-3 UPLC-Q-TOF-MS spectrum of peak b4 from TIC-UPLC-Q-TOF analysis result

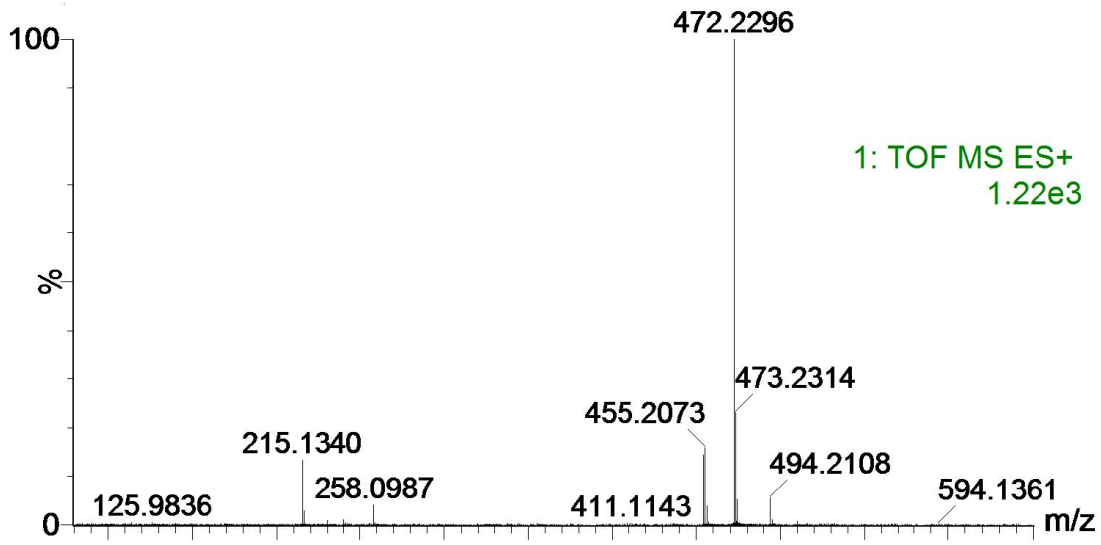


Fig. 2-4 UPLC -Q-TOF-MS spectrum of peak b3 from TIC-UPLC-Q-TOF analysis result

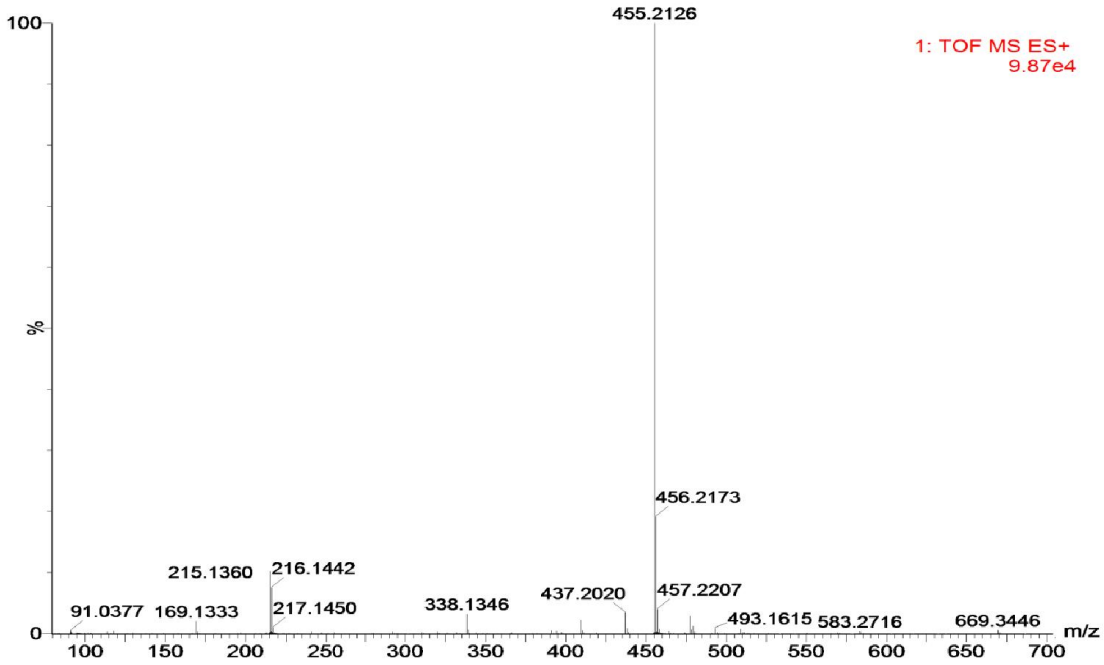


Fig. 3 Effects of QEPVL on NO release

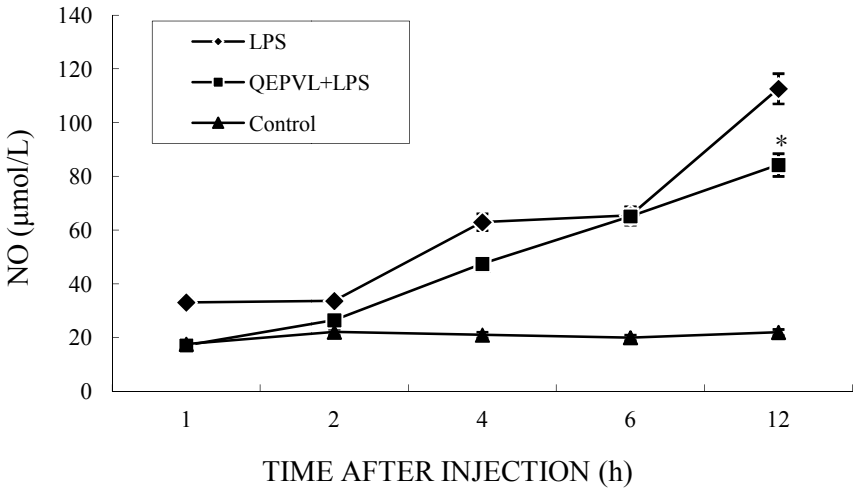


Fig. 4 Effects of QEPVL on cytokine expression

Fig.4-1 Effects of QEPVL on TNF-α production

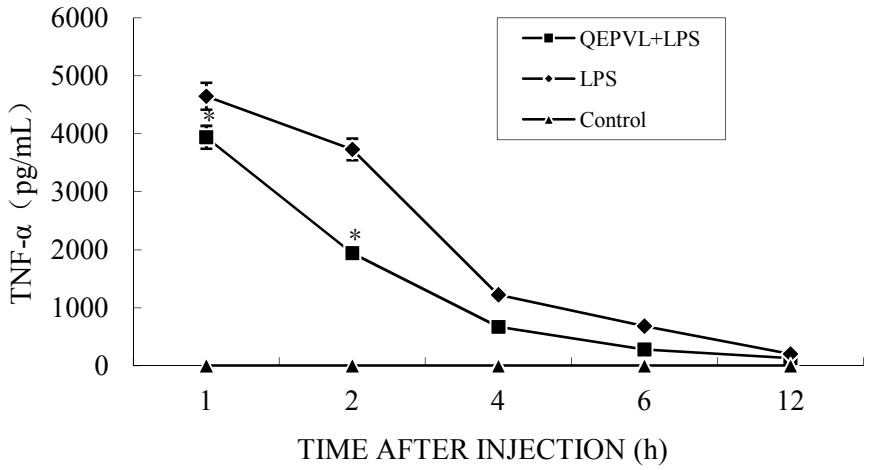


Fig.4-2 Effects of QEPVL on IFN-γ production

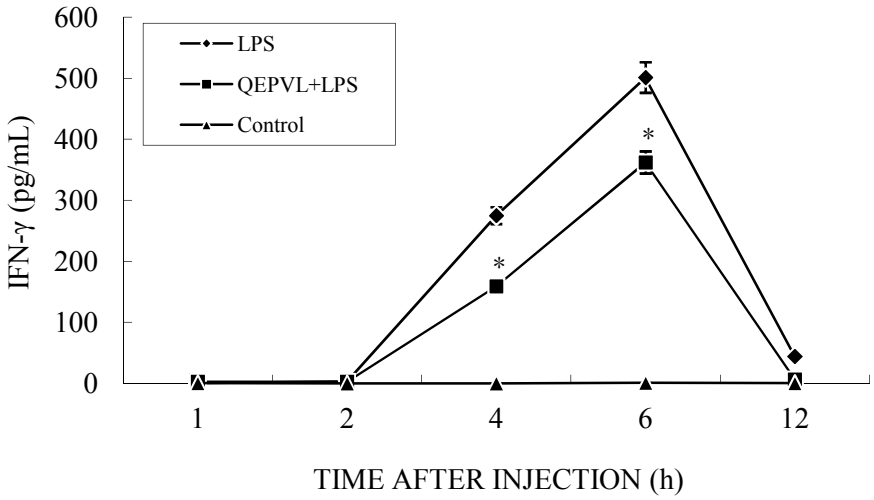


Fig.4-3 Effects of QEPVL on IL-4 production

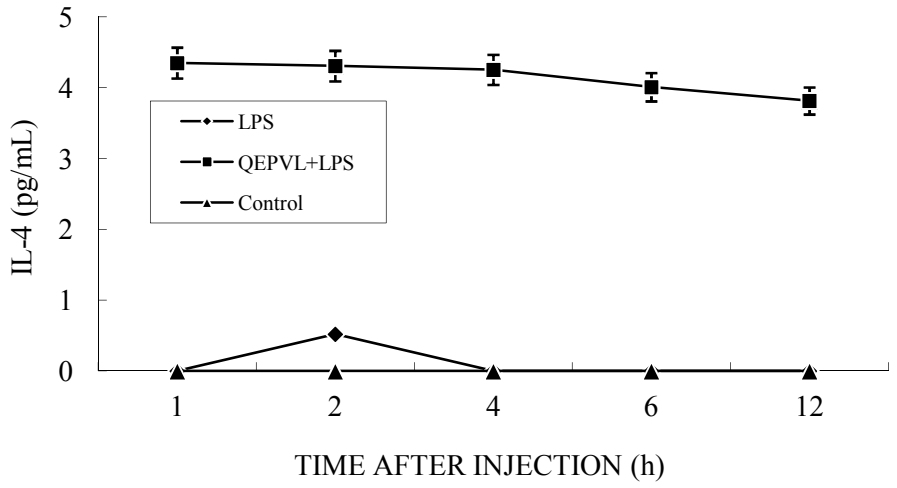
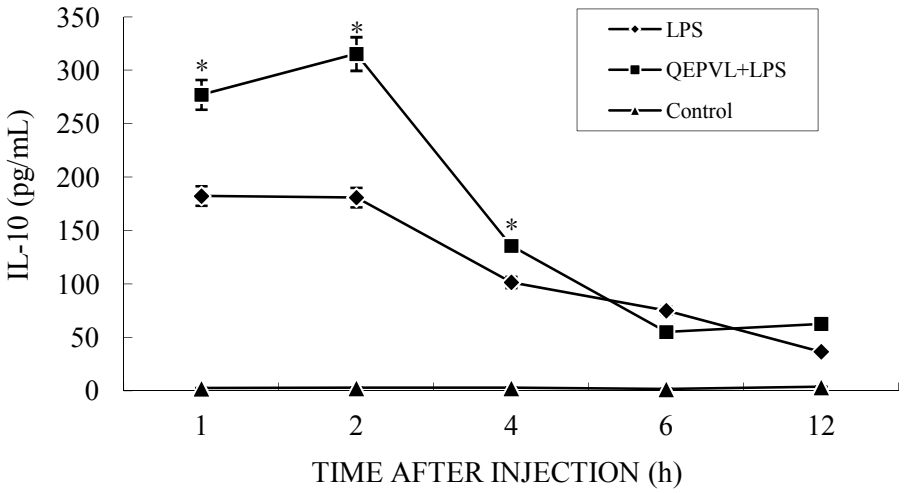


Fig.4-4 Effects of QEPVL on IL-10 production



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