

Effect of tyrosinase and caffeic acid crosslinking of turbot parvalbumin on the digestibility, and release of mediators and cytokines from activated RBL-2H3 cells

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ABSTRACT

Turbot can induce allergy in susceptible individuals due to the presence of parvalbumin (PV), a major fish allergen. This study aimed at evaluating the digestibility and the ability of PV to elicit the release of cellular degranulation, following treatment with tyrosinase (PV-Tyr), caffeic acid (PV-CA) and in combination (PV-Tyr/CA), using *in vitro* digestion and RBL-2H3 (passive rat basophil leukemia) cell line. The digestion assay products revealed that the stability of PV in simulated gastric fluid (SGF) was stronger, while in simulated intestinal fluid (SIF) was rather weak. Western blot analysis revealed that the IgG-binding abilities of the cross-linked PV were markedly reduced. Moreover, crosslinking hampered the release of cellular degranulation process in RBL-2H3 cell lines. PV-Tyr/CA showed highly significant reduction in the release rate of β -hexosaminidase (66.02%), histamine (35.01%), tryptase (29.25%), cysteinyl leukotrienes (29.72%), prostaglandin D₂ (34.96%), IL-4 (43.99%) and IL-13 (38.93%) and shown potential in developing hypoallergenic fish products.

1. Introduction

Fish is extensively consumed throughout the world that plays a major role in the human diet by providing highly assimilated proteins. Despite its rich nutritional value and delicious taste, it can also induce IgE-mediated allergic reaction upon ingestion by fish-allergic individuals (Rewnik & Owalski, 2016). It is among the big eight major food allergen responsible for eliciting allergic reactions (Canani et al., 2013). The common clinical symptoms include oral allergy syndrome, abdominal pain, rhinitis, urticaria, diarrhea, asthma, and angioedema, and in severe cases can even cause life-threatening anaphylaxis (Bock, Muñoz-Furlong, & Sampson, 2001; Rewnik & Owalski, 2016). Parvalbumin (PV), an EF-hand calcium-binding protein, is the main fish allergen that is responsible for causing allergy in 96–100% of fish-allergic patients (Rewnik & Owalski, 2016). It is a globular protein having a molecular weight of 12 kDa, which is heat-stable and water-soluble (Arif, 2009; Saptarshi, Sharp, Kamath, & Lopata, 2014). Carral, Martin-Lazaro, Ledesma, and De La Torre (2010) found that ingestion of turbot proteins caused asthma in farm workers, with parvalbumin as one of the causing agents.

The immunoreactivity of allergens depends on the retention of their native structural epitopes and their tendency to be recognized by immunoglobulin G (IgG) or immunoglobulin E (IgE) antibodies

(Verhoeckx et al., 2015). Therefore, several thermal and non-thermal processing methods are adapted to lessen the allergenic potential of proteins by altering their structural epitopes (Ahmed et al., 2018; Lv et al., 2018; Lv, Lin, Li, Wang, Ahmed, & Chen, 2017a; Lv, Lin, Li, Ahmed, & Chen, 2017b; Khan et al., 2018; Yuan et al., 2018). Crosslinking of food protein via enzymatic treatment possesses a great prospect to tailor food functionality due to the specificity of enzymes and mild reaction conditions. Protein crosslinking is the process of amalgamation of protein molecules through intra- or inter-molecular covalent bonds (Heck, Faccio, Richter, & Thöny-Meyer, 2013). Currently, food industries utilize various enzymes (i.e., tyrosinase (Tyr), laccase, peroxidase and transglutaminase) having the capability to form covalent crosslinks in proteins. These enzymes vary from each other in term of the reaction mechanism and the amino acid residues with which they react (Isaschar-Ovdat & Fishman, 2017; Selinheimo, Lampila, Mattinen, & Buchert, 2008; Strong & Claus, 2011). Tyrosinase is a copper-containing oxidative enzyme that catalyzes the ortho-hydroxylation of monophenols to o-diphenols and on subsequent oxidation converts to quinones. These quinones are very reactive, so they further react with other amino and sulfhydryl groups (Bittner, 2006; Selinheimo et al., 2008). Cura et al. (2010) reported crosslinks formation in heated milk after the incorporation of Tyr. Moreover, Ahmed et al. (2018) cross-linked shrimp TM via Tyr and caffeic acid (CA) treatments and observed

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changes in the conformational structure and physicochemical properties. These modifications further reduced the IgE/IgG binding capacities and assisted in the development of the hypoallergenic aquatic products. So based on these studies, it was hypothesized that Tyr/CA aided crosslinking could mitigate the allergenicity of turbot PV in the gastrointestinal digestion and the RBL-2H3 cell line.

The stability of food allergens in gastrointestinal digestion, as well as the degranulation rates in basophils and mast cells are the key factors in determining the immunoreactivity of allergens. For eliciting an allergic response, the integrity of allergen epitopes needs to remain undamaged in the gastrointestinal tract (Lv, Lin, Li, & Ahmed et al., 2017; Lv et al., 2019; Perera et al., 2010). However, susceptible individuals can be exposed to pure allergens very occasionally. Food processing and the presence of other food matrix components may alter the stability of allergenic protein during gastrointestinal digestion. Besides, basophils and mast cells are the key factors for triggering IgE-mediated allergic reactions. Previously, several research studies have shown that these cells play an important role for diagnosing allergic responses during *in vitro* assays (Erdmann, Heussen, Moll-Slodowy, Merk, & Sachs, 2003; Perera et al., 2010). Regardless of the fact, the collection of mast cells and basophils are difficult owing to human subjects and ethical considerations. Therefore, RBL-2H3 cells are used as alternative and reliable models for studying allergic response at cellular level, as these are mucosal mast cells comparable to primary mast cells and common basophils (Liu et al., 2017; Passante & Frankish, 2009; Song et al., 2010).

In order to better understand the effect of Tyr and CA on turbot PV, it is necessary to evaluate the digestive stability, and allergenicity changes on the effector cells of the IgE mediated allergy. Therefore, the aim of this study was to compare the digestive stability and allergenicity of cross-linked PV in gastrointestinal fluids and RBL-2H3 cell line. Simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) was carried out to observe the stability of native and cross-linked PV. Additionally, in RBL-2H3 cell line, the allergenicity changes of cross-linked PV was evaluated by determining the release of β -hexosaminidase, histamine, cysteinyl leukotrienes, prostaglandin D₂, IL-4 and IL-13 levels. Thus, this study provides an insight to understand the potential of these treatments in minimizing the risk of allergies associated with fish products, thereby improving the life quality of allergic people.

2. Materials and methods

2.1. Materials and reagents

Mushroom Tyr and CA were acquired from Sigma Chemical Co. (St. Louis, MO, USA) and Shanghai Yuanye Co. (Shanghai, China), respectively. Turbot (*Scophthalmus maximus*) was purchased alive from the local fish market of Qingdao. The protein marker (Cat No. PR1900), loading buffer and Coomassie Brilliant Blue R-250 Staining Solution for performing SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) were procured from Solarbio Co. (Beijing, China). The enhanced chemiluminescent (ECL) kit for Western blot analysis was procured from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Rabbit polyclonal IgG antibody against PV was generated by our lab. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody (Cat No. SE101) and anti-human IgE antibody (Cat No. AS10754) were purchased from Solarbio Co. (Beijing, China) and Agrisera (Vannas, Sweden), respectively. The trypsin and porcine pepsin were obtained from Sigma (St. Louis, MO, USA). Advanced Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco Life Technologies (Grand Island, NY, USA). ELISA kits for histamine, tryptase, cysteinyl leukotriene, prostaglandin D₂, IL-4, and IL-13 were purchased from R & D Systems (Minneapolis, MN, USA). All other reagents used in the experiments were of analytical grade unless stated otherwise.

2.2. Patient sera

Sera were obtained from four patients allergic to fish from the Affiliated Hospital of Medical College of Qingdao University, Qingdao. These patients had a documented clinical history of allergy caused by fish and were confirmed as fish allergy on the basis of specific IgE level higher than 0.35 kU/L and positive skin prick test to parvalbumin, as determined by Allergy Screen (MEDIWISS Analytic GmbH, Moers, Germany). Sera were collected and informed consents were signed by each patient stating that they are fully aware of the intended use of their sera. The sera were stored at -80°C until use. The protocol has the approval of the local medical ethics committee (File No. QD-PF-20140314551) in order to protect the interests and rights of the human subjects.

2.3. Purification of turbot PV

Live fresh turbot was brought to the laboratory and killed by stunning. The fish skin, bones and internal organs were removed after slaughtering. The spine meat was taken and homogenized until the meat was mixed uniformly. The flesh of the turbot was extracted at 4°C overnight using extraction solution (Tris 0.1 M/L; Glycine 0.5 mM/L; DTT 0.1 mM/L). The mixture was centrifuged at ($1070\times g$) for 30 min to obtain a supernatant, which was then added to a 3.5 kDa dialysis bag for dialysis overnight to obtain a whole protein. Afterward, 50 mL of total protein was taken and centrifuged at ($8120\times g$) for 30 min. After the collection of supernatants, ammonium sulfate was slowly added to the sample to the saturation of 60%, and incubated at 4°C overnight. Again, the extracts were centrifuged at ($5210\times g$) for 30 min, followed by a collection of the precipitate after the addition of a small amount of ultrapure water. Later, the precipitates were reconstituted and dialyzed overnight and the resulting solution was lyophilized, and stored at -40°C until further use. The purity of native PV was analyzed using SDS-PAGE.

2.4. Tyr and CA assisted crosslinking of turbot PV

The activity of mushroom Tyr was measured following the method described earlier by Yang and Ouyang (2012). The conditions of enzymatic crosslinking reactions were decided based on the previous study (Thalmann & Lötzbeyer, 2002) and pre-experiments. Fish PV was prepared at a concentration of 2.0 mg/ml in 10 mM phosphate-buffered saline (PBS, pH 6.5) and incubated with Tyr (600 U/g of PV) and caffeic acid (0.1 mg/mg PV). All samples were incubated under constant agitation for 12 h at 37°C , followed by boiling the samples for 5 min to stop the reaction. Control samples that lack Tyr or CA were also run in parallel with the test samples. All the samples were kept at -20°C until further analyses.

2.5. In vitro digestion assay of native and cross-linked PV

2.5.1. SGF digestion stability assay

The digestibility assay of Tyr and CA treated PV was determined as described by Huang et al. (2010) with minor modifications. The SGF was made as illustrated in the United States Pharmacopoeia (1995) that comprised of porcine pepsin (275 U/mg protein) in 34.3 mM NaCl and in an acidic pH 1.2. The reaction solution volume was made to 1 mL. The SGF (1:10.4 pepsin to protein, w:w) was preheated for 15 min at 37°C before the test protein addition for each sample. The digestion was carried out with continuous shaking at 37°C . 90 μL of the reaction solution was added to a separate sampling tube at a different time interval (0, 1, 2, 5, 10, 15, 30 and 60 min) and the reaction was stopped by the addition of 30 μL 0.2 M Na₂CO₃. The samples were heated at a boiling temperature for 7 min followed by analyzing with SDS-PAGE and western blot. The protein samples for the 0 min time point were added to SGF, which was neutralized with Na₂CO₃. For the control,

each sample was mixed with the reaction mixture exclusive of pepsin, followed by incubation at 37 °C for 1 h. The experiment was carried out in triplicate to obtain uniformity.

2.5.2. SIF digestion stability assay

The SIF for intestinal digestion was also prepared by following the United States Pharmacopoeia (1995), and comprised of trypsin in 5 mM KH_2PO_4 (pH 7.5). The total reaction solution was set to a volume of 1 mL. The enzymatic assay of trypsin was 162 U/mg proteins. Trypsin to protein ratio was 1:50 (w:w) and the digestion was performed with incessant shaking at 37 °C. The reaction solution (100 μL) was transferred to another sampling tube at each listed time point (0, 1, 15, 30, 60, 120, 180 and 240 min). The reaction was terminated by heating at 95 °C for 5 min. In case of control, no trypsin was added with the PV samples in the reaction solution. The samples were then analyzed by SDS-PAGE and western blotting.

2.5.3. SDS-PAGE and Western blot analyses of native and cross-linked PV

The protein samples were analyzed by SDS-PAGE, as described by Laemmli (1970). All the samples (~6 μg of protein) were mixed with a loading buffer and heat-denatured at 100 °C for 7 min. Then the samples were loaded into an SDS-PAGE gel composed of a 5% stacking gel and a 15% running gel. A precision protein standard (14–180.0 kDa) was loaded in the first well of the gel as a molecular weight marker. The samples were run through the stacking gel at 80 V and through the resolving gel at 120 V, followed by staining with 0.1% Coomassie brilliant blue R-250 dye. After de-staining, images were captured by HP Scanjet G4050 Photo Scanner (Hewlett-Packard Co., USA).

Western blot analysis was carried out to assess the IgG binding potentials of all the cross-linked PV samples according to the method described earlier by Ahmed et al. (2018) with some modifications. In brief, samples were electrophoresed and the pre-run gel was transferred to a polyvinylidene fluoride (PVDF) membrane of the iBlot® 2 Gel Transfer Device (Life Technologies Co., Israel). IgG rabbit polyclonal antibody (1:1500 dilution, v/v; 2 mL) and HRP-labeled goat anti-rabbit IgG (1:2000 dilution, v/v; 2 mL) antibody were prepared with the iBind solution. Later on, the immuno-detections were carried out using iBind™ Western Device (Life Technologies Co., Israel). Following that, the antibody-bound protein on the blot was photographed by Tanon-4200SF gel imaging system (Tanon Science & Technology Co., Ltd, Shanghai, China) using an ECL substrate. Multiple exposure times were given to obtain an optimal image.

2.6. Allergenic changes of native and cross-linked PV in RBL-2H3 cell lines

2.6.1. Cell culture

The RBL-2H3 cells were acquired from the Chinese Academy of Sciences and were grown according to the method of Song, Li, Gao, Pavase, and Lin (2016). Initially, the cells were cultured in a minimum essential medium DMEM with the supplementation of 10% FBS, streptomycin (100 $\mu\text{g}/\text{mL}$) and penicillin (100 U/mL). Next, the cells were incubated with a 5% CO_2 / 95% air atmosphere at 37 °C.

2.6.2. Mediator and cytokine release

For the measurement of mediator and cytokine release, RBL-2H3 cells were collected at the stationary stage, followed by seeding in a 96-well microtitre plate. After that, human serum IgE was added for passive sensitization and incubated for 24 h. After washing three times with Tyrode's buffer, then the cells were stimulated with 0.1 mg/mL PV and cross-linked PV. The cells treated with only Tyrode's buffer were used as negative control. The PV concentration was optimized by preliminary titrations in DMEM to prompt higher release of β -hexosaminidase. The release of β -hexosaminidase was measured according to the method described earlier (Kuehn et al., 2010; Lv et al., 2018). Firstly, RBL-2H3 cells were stimulated with native and cross-linked PV (500 ng/mL) in Tyrode's buffer for 60 min at 37 °C. Cells were

incubated for 60 min in order to determine the release of histamine, cysteinyl leukotriene, prostaglandin D₂, IL-4 and IL-13. After the incubation period, the samples were centrifuged at 3000 $\times g$ and 4 °C for 10 min to pellet. The remaining cell debris and granules were discarded. While, the supernatants of the cell culture were collected and analyzed via ELISA assay kits following the manufacturer's instructions.

2.7. Statistical analysis

Data were depicted as mean $\pm$ standard deviation (SD) of three independent experiments. Differences among experimental data were analyzed by an analysis of variance (ANOVA) using the SPSS Statistics 18 software (SPSS Inc., Chicago, USA). Statistical significance of each test was defined at $p < 0.05$.

3. Results

3.1. SDS-PAGE analysis of native and cross-linked PV by SGF

In this study, we performed Tricine-SDS-PAGE for analyzing the changes in protein components of Tyr and CA-treated PV following digestion (Fig. 1). The results indicated that the digestive patterns of PV samples by pepsin were very different (Fig. 1). As shown in Fig. 1A, turbot PV has a weak stability to simulated gastric fluid. After 1 min, the PV was more obvious, almost undigested, and a small molecular band was formed at 6 kDa. As the reaction progressed, the native PV bands almost disappeared, and the proteolytic fragment at 6 kDa began to be digested and degraded, resulting in fuzzy band groups at 3–5 kDa. PV-Tyr showed enhanced SGF digestion stability (Fig. 1B), however, the PV bands were weakened due to the high molecular weight dimers formation after crosslinking. Crosslinking of PV by CA also enhanced the SGF stability compared to the control group, and the bands produced by crosslinking gradually became shallower and disappeared at 60 min of digestion (Fig. 1C). Fig. 1D shows the SGF digestion pattern of PV-Tyr/CA and the crosslinking efficiency of Tyr was significantly enhanced in the presence of CA. The PV bands have been quite discreet and almost disappeared by forming a “tailing” at above 12 kDa.

3.2. SDS-PAGE analysis of native and cross-linked PV by SIF

As shown in Fig. 2A, PV has higher digestion stability in SIF, and the original protein band has almost no changes in the initial stage of digestion. After 1 min of digestion, new bands were produced at 4 and 6 kDa respectively, and then these bands markedly reduced as the enzymatic digestion progressed, indicating that the small molecular products were preferentially digested in the SIF. After 240 min, the original protein band was still very clear, and the small molecule products were almost digested. On the other hand, the crosslinking of turbot protein with Tyr simulated the SIF digestive stability, accompanied by the production of cross-linked products. As shown in Fig. 2B, the digested PV-Tyr produced a large number of small molecule products after a short time of digestion. In the first 60 min, the SIF mainly digested the newly formed small molecule products and the PV dimers produced due to the crosslinking reaction. After 60 min, the original PV band began to become lighter, while at 120 min, the 12 kDa band was almost invisible. Compared with PV-Tyr, CA aided crosslinking has a weaker effect on digestion and produced a new band at the lowermost end of the electrophoresis gel after 180 min and 240 min (Fig. 2C). As shown in Fig. 2D, the PV cross-linked by Tyr and CA was not obvious similar to SGF, and the dispersion was intense. The SIF digestion affected these cross-linked products with uncertain molecular weight.

3.3. Western blot analysis of native and cross-linked PV by SGF and SIF

Western blot analysis was carried out to analyze the immunological activities of the native and treated PV as well as the proteolytic

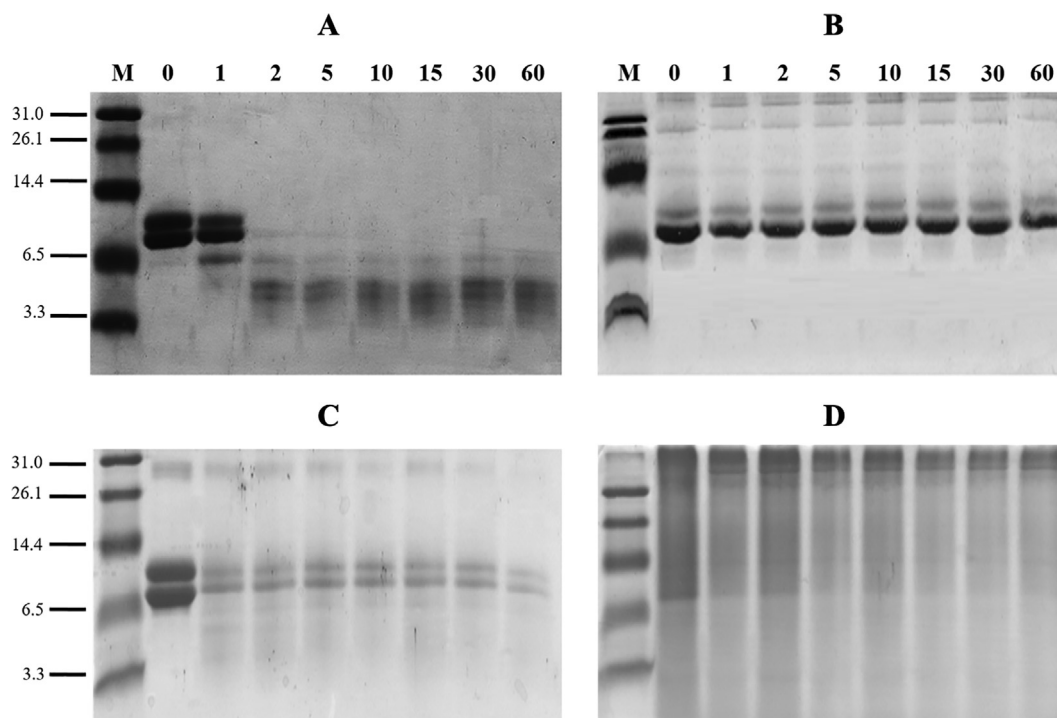


Fig. 1. Effect of simulated gastric fluid (pepsin) digestion on turbot parvalbumin (PV). (A) untreated PV; (B) PV crosslinked by Tyrosinase (PV-Tyr); (C) PV crosslinked by Caffeic acid (PV-CA); (D) PV crosslinked by Tyrosinase and Caffeic acid (PV-Tyr/CA). Peptic digestion was performed, followed by Tricine-SDS-PAGE and were visualized by staining with Coomassie brilliant blue dye. The protein content was 6 μ g per lane. Lane M – Pre-stained Protein Marker 3.3–31.0 kDa (Beijing, China); lanes 0–60 – fluid collection along digestion process (min).

fragments at different time points during SGF and SIF digestions. Fig. 3A–D shows the IgG binding ability of PV and cross-linked PV after SGF digestion, and the results are similar to those of electrophoresis. After digestion, the IgG binding ability of PV and treated PV rapidly

decreased. Tyr cross-linked PV maintained its IgG binding ability for 10 min, and was almost lost after 15 min (Fig. 3B). Similarly, the CA cross-linked PV showed no significant changes following digestion, and the newly formed products were still recognized by IgG during the first

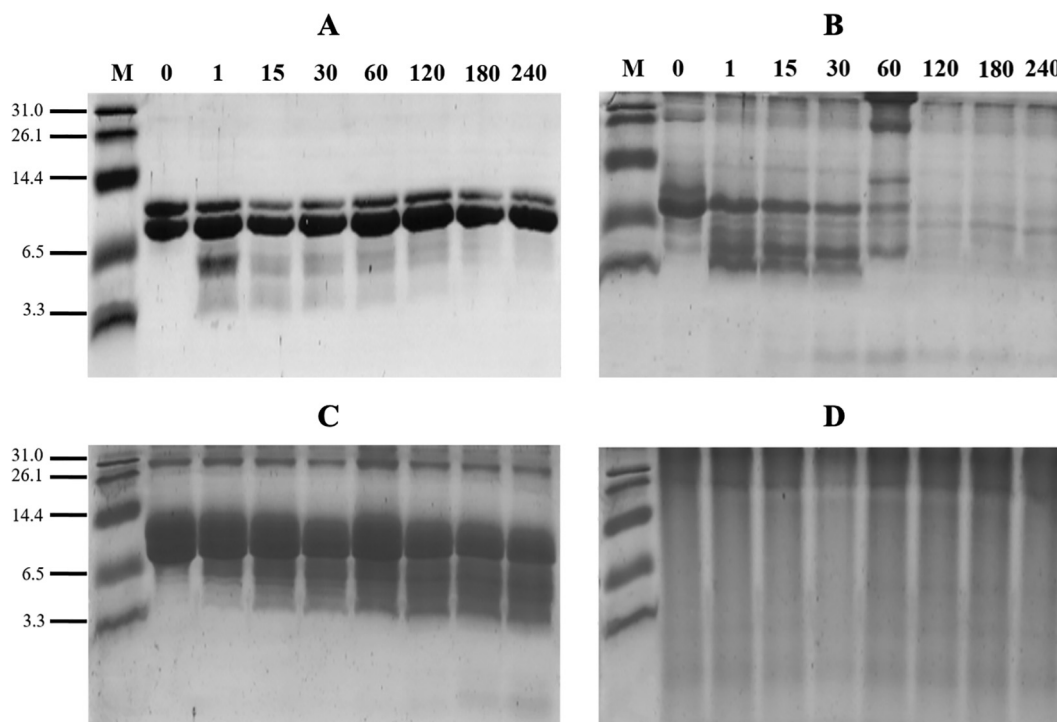


Fig. 2. Effect of simulated intestinal fluid (trypsin) digestion on turbot parvalbumin (PV). (A) untreated PV; (B) PV-Tyr; (C) PV-CA; (D) PV-Tyr/CA. Tryptic digestion was performed, followed by Tricine-SDS-PAGE and were visualized by staining with Coomassie brilliant blue dye. The protein content was 6 μ g per lane. Lane M – Pre-stained Protein Marker 3.3–31.0 kDa (Beijing, China); lanes 0–240 – fluid collection along digestion process (min).

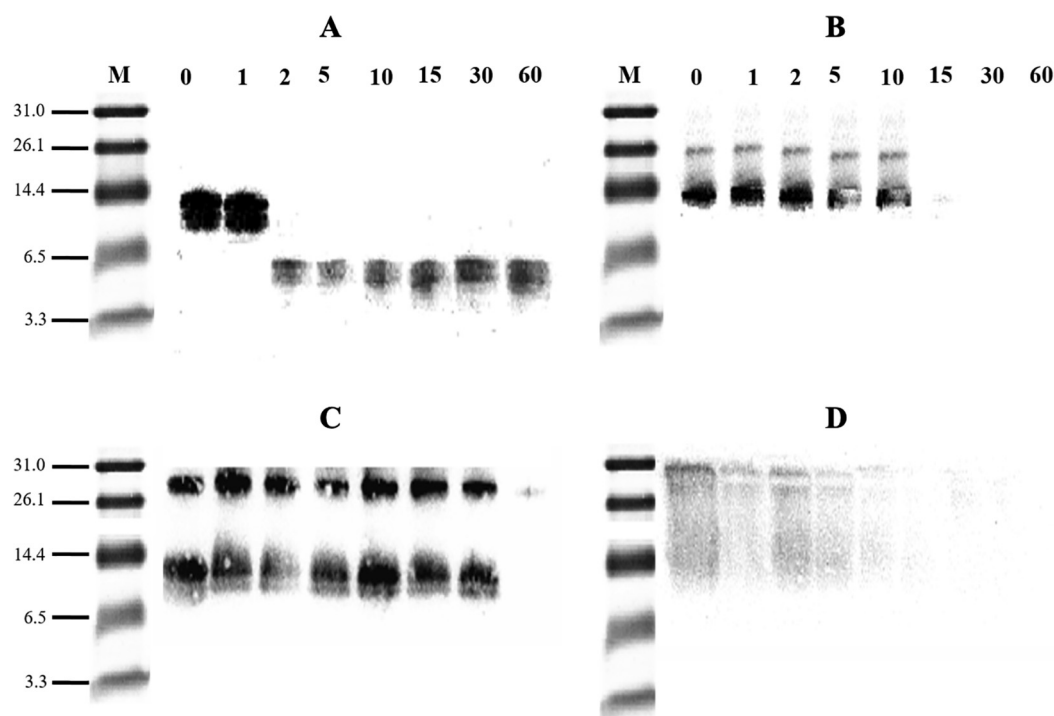


Fig. 3. Western blot detection of the degradation of PV and cross-linked PV in SGF digestion fluids. Samples were separated by SDS-PAGE followed by immunological detection using rabbit polyclonal antibodies against PV. (A-D) Western blotting of SGF digestion of native PV, PV-Tyr, PV-CA, and PV-Tyr/CA, respectively. Lane M – Pre-stained Protein Marker 3.3–31.0 kDa (Beijing, China); lanes 0–60 – IgG binding capacity of native and treated PV during the digestion process (min).

30 min. While the PV bands were disappeared at 60 min, and lost IgG binding ability (Fig. 3C). It is worth noting that crosslinking of PV by Tyr and CA further reduced the antigenicity and the IgG binding capacity of the PV and digested products were disappeared after 15 min of digestion (Fig. 3D).

The immunological activity of the PV after SIF digestion showed no

changes and the 12 kDa protein band was clear and distinct (Fig. 4A). Trypsin digestion experiments showed that the cross-linked PV samples were more easily digested and degraded, resultantly led to the reduction of immunological activities. At the start of digestion, the changes in the IgG binding ability was not significant within 1 min, and the binding ability was substantially decreased with the progression of

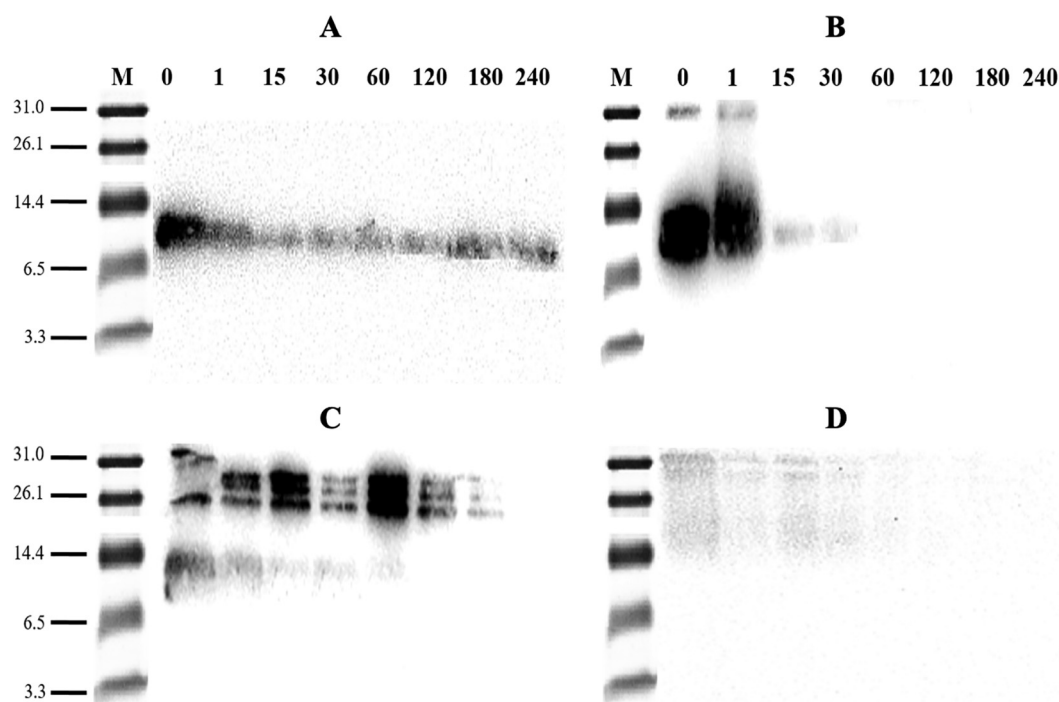


Fig. 4. Western blot detection of the degradation of PV and cross-linked PV in SIF digestion fluids. Samples were separated by SDS-PAGE followed by immunological detection using rabbit polyclonal antibodies against PV. (A-D) Western blotting of SIF digestion of native PV, PV-Tyr, PV-CA, and PV-Tyr/CA, respectively. Lane M – Pre-stained Protein Marker 3.3–31.0 kDa (Beijing, China); lanes 0–240 – IgG binding capacity of native and treated PV during the digestion process (min).

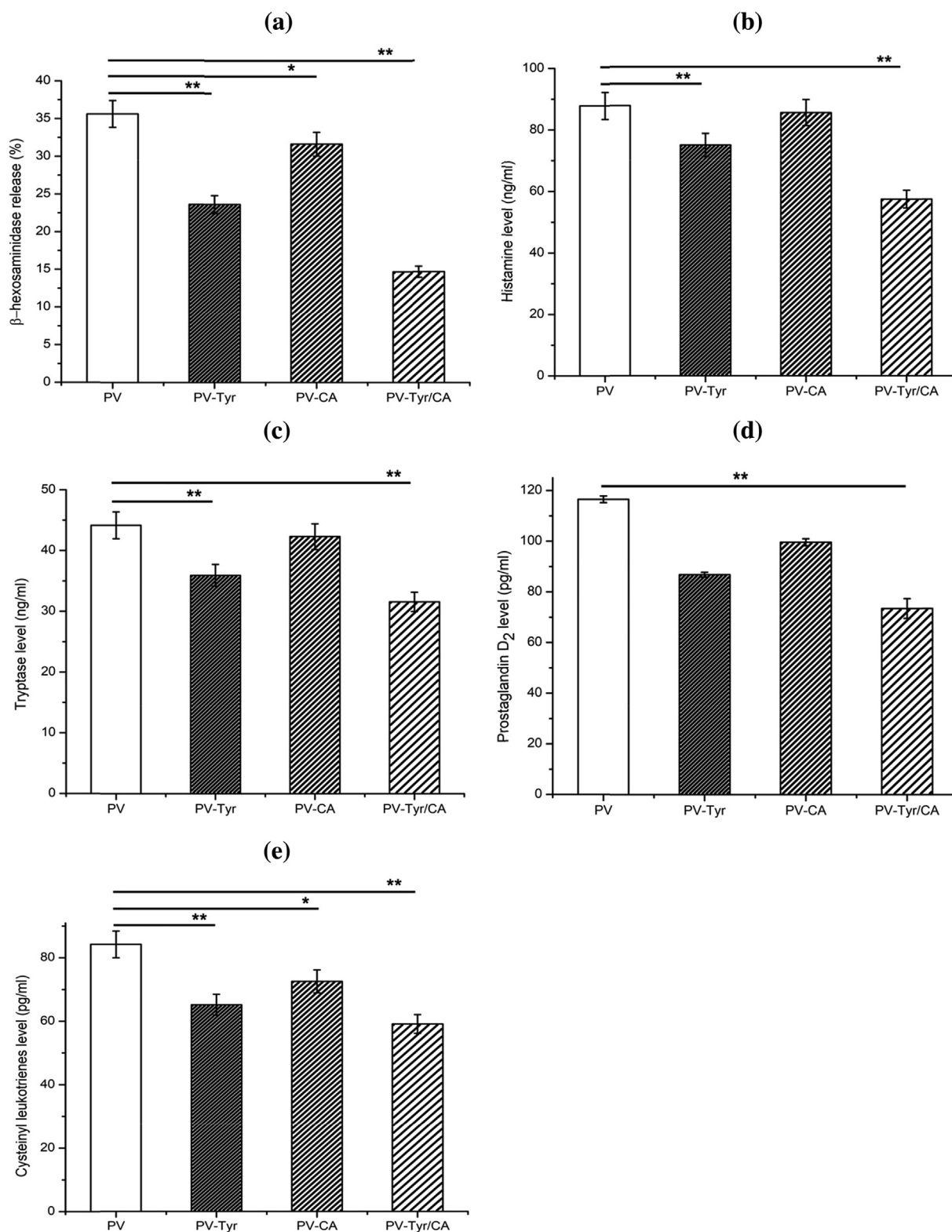


Fig. 5. (a) β -hexosaminidase, (b) histamine, (c) tryptase, (d) prostaglandin D₂, and (e) cysteinyl leukotriene release from RBL-2H3 cells sensitized with sera from fish allergic patients and challenged with native or cross-linked PV. The data represent the mean $\pm$ SD of three independent experiments. *, p < 0.05; **, p < 0.01.

digestion time. After 15 min, the IgG binding capacity of cross-linked PV has been greatly reduced, while the binding to PV were almost vanished within one hour (Fig. 4B-D). The results of SIF digestion of treated PV are similar to those of SGF digestion and is obvious that the IgG binding ability has a substantial reduction effect.

3.4. Mediator release assay of native and cross-linked PV

In this study, RBL-2H3 cells sensitized with fish allergic patient sera were used to evaluate the effects of Tyr and CA-induced crosslinking on the allergenicity of turbot PV. The release of mediators, namely: β -hexosaminidase, histamine, tryptase, prostaglandin D₂, and cysteinyl

leukotriene are shown in Fig. 5. The release rate of β -hexosaminidase is positively correlated with the increase in the concentration of PV (Fig. S1, supplementary material). The release rate began to stabilize as the concentration was 0.1 mg/mL, therefore, this concentration was selected to excite cells. As shown in Fig. 5a, the release of β -hexosaminidase by the cells was significantly reduced, namely 33.81% for PV-Tyr, 16.53% for PV-CA, and 66.02% for PV-Tyr/CA, compared to the control group. This indicates that the crosslinking reaction can effectively inhibit the phenomenon of degranulation, thereby alleviating the risk of allergies. Compared with the control group, the histamine content of the treated PV decreased from 87.79 ng/mL to 75.00, 85.52 and 57.42 ng/mL, respectively, with a decrease of 18.32%, 2.61% and 35.01% (Fig. 5b). While, tryptase content reduced by 15.41%, 4.21% and 29.25% (Fig. 5c); the content of prostaglandin D2 reduced by 24.79%, 12.68% and 34.96% (Fig. 5d); and the content of cysteinyl leukotriene decreased by 22.53%, 13.82% and 29.72% (Fig. 5e), respectively. This indicates that Tyr aided crosslinking in the presence of CA can significantly inhibit the release of active mediators by PV.

3.5. Cytokine release of native and cross-linked PV

Interleukin-4 (IL-4) and IL-13 were measured to illustrate the effect of Tyr and CA treatments, either alone or in combination, on turbot PV. The IL-4 content of the treated samples decreased from 72.09 ng/mL to 59.57, 69.81 and 40.62 ng/mL, respectively, with a reduction of 17.85%, 3.73%, and 43.99%, respectively, compared to the control group (Fig. 6a). While, the percent reduction in case of IL-13 contents were 25.02%, 11.11% and 38.93%, respectively. It indicates that the crosslinking of PV using combined treatment of Tyr and CA can effectively reduce the release of cytokines. Although Tyr and CA alone also have certain effects on the cellular degranulation process, though the reduction rate was not very pronounced.

4. Discussion

In the present study, we examined the digestibility of PV, PV-Tyr, PV-CA and PV-Tyr/CA in simulated gastrointestinal fluids. The digestion time for SGF and SIF were assigned (1 and 4 h, respectively) based on the time factor an ingested food normally remains in the human digestive system. In the SGF digestion, the stability of PV was poor, presumably because of the first specific degradation of Phenylalanine and Tyrosine residues by pepsin, due to their relatively high contents (Astwood, Leach, & Fuchs, 1996). As observed, the digestive patterns of PV samples by pepsin and trypsin were very different, which are due to

the variation in the cleavage specificities of the two proteases. Generally, pepsin offers a higher cleaving specificity for peptide bonds and Phenylalanine/Tyrosine residues (Mikita & Padlan, 2007). On the other hand, trypsin cleaves hydrophilic amino acids such as Lysine and Arginine residues (Barrett, Rawlings, & Woessner, 2004; Huang et al., 2010).

The results of immunoblotting were different from those of electrophoresis, indicating that the immunological activity of the PV after crosslinking decreased significantly. The reason is that crosslinking led to structural changes of PV, and some antigen-binding sites might be exposed on the surface, which were destroyed after being digested by gastrointestinal fluid. Crosslinking reaction can alter structure and hydrophobicity of allergens and a correlation was found between these changes and gastrointestinal digestion in recent studies (Liu et al., 2017; Lv et al., 2019; Xie, Wang, Jiang, Liu, & Li, 2015; Zhou, Zhao, Cui, & Sun, 2015). Crosslinking induced loose networks and resultantly led to increased digestibility by providing more cleavage sites. In addition, the new small fragments did not have IgG-binding activity. This eventually elicited a different immunological response compared to the native PV. Untersmayr et al. (2005) revealed that codfish PV began to decompose within 1 min under simulated gastric fluid digestion conditions and showed a reduction in the immunoreactivity that was reliant on the digestion time. Fei et al. (2016) conducted a similar study on the arginine kinase of the blue crab, and the results showed that the stability of peptidase digestion of arginine kinase after enzyme crosslinking was enhanced and the immunoreactivity of the allergenic protein was decreased to some extent. The treatment with proteases, especially trypsin, is quite effective in decreasing the IgG-binding capacity of turbot PV. The smaller proteolytic fragments generated by SGF and SIF digestion in case of cross-linked PV showed no binding to IgG as the digestion period prolonged. Tyr or CA mediated crosslinking promoted the digestion of PV and effectively reduced its IgG binding ability, indicating that the immunological activities of the degradation products have been destroyed to a greater extent.

Furthermore, the sensitizing ability of turbot PV following Tyr and CA crosslinking was determined by causing a degranulation reaction in RBL-2H3 cell lines. Mast cells and basophils play a major role in IgE-mediated allergy because food allergens can activate mast cells and basophils to cause degranulation. In type I allergy, degranulation occurs in basophils releasing substances by exocytosis, including active mediators and cytokines, thereby eliciting the corresponding allergic symptoms (Passante & Frankish, 2009). In the present study, crosslinking of PV resulted in the suppression of the release of mediators related to allergic symptoms. Likewise, Song et al. (2016) observed a

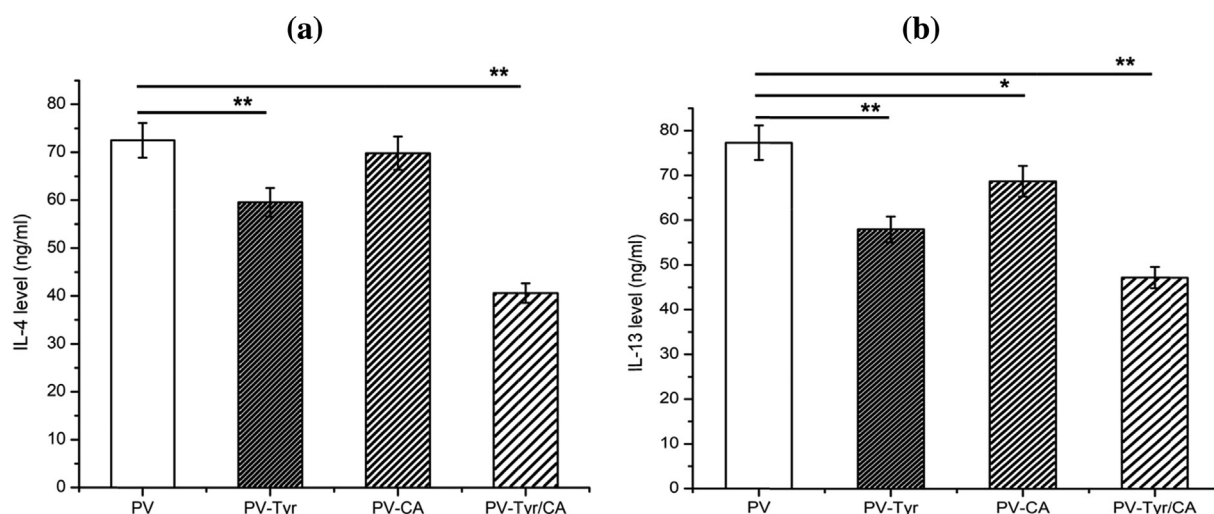


Fig. 6. IL-4 and IL-13 release from RBL-2H3 cells sensitized with sera from fish allergic patients and challenged with native or cross-linked PV. The data represent the mean $\pm$ SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

reduction in the ability of shrimp tropomyosin to induce allergen mediator (i.e., β -hexosaminidase, histamine, tryptase, prostaglandin D₂, and cysteinyl leukotriene) and cytokine (i.e., IL-4 and IL-13) release in RBL-2H3 cells upon malondialdehyde treatment.

The present finding indicated that Tyr modified PV has lower potential to secrete IL-4 and IL-13. Cytokines can regulate allergic reactions, for example, IL-4 can directly act on mast cells, leading to intestinal dilatation and enhancing allergic reactions. However, inhibition of the degranulation reaction can suppress the occurrence of allergic reactions (Choi & Yan, 2009). More recently, Lv et al. (2019) treated shrimp tropomyosin with 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and observed a decrease in the secretion of mediators and cytokines in RBL-2H3 cells. Toda et al. (2011) reported that protein unfolding could significantly mitigate the potential sensitization of the peach allergen Pru p3 and inhibit the mast cell degranulation reaction. The proposed explanation was that the response of RBL-2H3 cells to the cross-linked allergen might be because of the altered binding epitopes on the surface of Tyr-treated PV, hindering successful basophil degranulation. Modification of protein may alter its allergenic potential at the cellular level due to changes in the binding epitope and other associated conformational structures. Recently, Tian et al. (2019) observed structural changes in turbot PV following crosslinking by Tyr and CA, which further reduced the IgG binding ability, as observed via western blotting and enzyme-linked immunosorbent assay. However, Iwan et al. (2011) showed that IgE binding capacity of the main allergen Cor a 11 decreased after heat treatment combined with Maillard reaction, but not the degranulation capacity of basophils. This suggests that not all processing methods can reduce the immunological activities of allergens at the cellular level. Allergic symptoms occur based on the capacity of release of the active mediators, therefore their measurement are more valued in order to comprehensively evaluate the state of the body in an allergic reaction.

5. Conclusion

In conclusion, the study has shown that Tyr and CA treatments of turbot PV resulted in high molecular weight covalently cross-linked proteins. Cross-linked allergens have a grossly reduced ability to trigger a gastrointestinal allergic reaction following peptic and tryptic degradation. Tyr and CA-treated PV significantly enhanced the digestion of PV and effectively reduced its immunoreactivity. In addition, Tyr/CA-cross-linked PV significantly decreased the release of cellular mediators and cytokines in RBL-2H3 cell line by inhibiting degranulation. Therefore, this study helps to illustrate the impact of enzymatic treatment on fish allergen during gastrointestinal digestion and at cellular level. Further research studies should be designed to study the effect of these treatments on the antigen uptake and also clinical trials are needed for validation.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.125209>.

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Allergenicity of tyrosinase and caffeic acid-treated turbot (*Scophthalmus maximus*) parvalbumin evaluated using *in vitro* digestion and RBL-2H3 cell line

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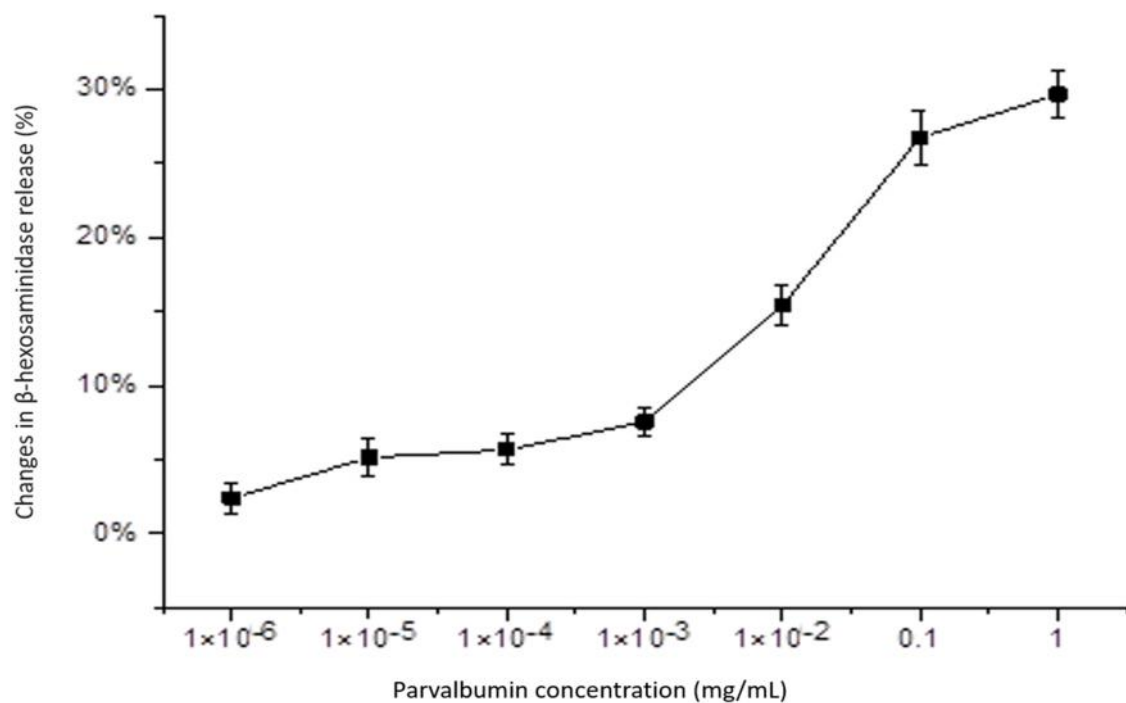


Figure S1: Changes of β -hexosaminidase release from sensitized RBL-2H3 cell line stimulated by different concentrations of PV.