





## Protein extract of *Detam 1* soybean contains $\beta$ -conglycinin stimulating cholecystokinin signal transduction through activities of PKC, c-RAF and ERK 1/2 in Wistar rats

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### ABSTRACT

**Objective:** to determine which kind of soybean extract contains the highest  $\beta$ -conglycinin level, their effects to cholecystokinin (CCK) plasma level, and to signal transduction pathway in duodenum of Wistar rats.

**Methods:**  $\beta$ -conglycinin were analysed in vitro using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and coomassie brilliant blue (CBB) staining. The CCK plasma level were measured in vivo in 24 male Wistar rats by ELISA method, using t-test paired and the activity of PKC $\mu$ , PKA, c-Raf and ERK in CCK signal transduction pathway in vivo by Western blot method and Scion image densitometry (DU) analysed using ANOVA.

**Result:** Protein extract of *Detam 1* soybean (PEDS) contains the highest amount of  $\beta$ -conglycinin level. There was no significant difference of CCK plasma level between pre and post treatment ( $p > 0.05$ ) although there were increasing level in all treatment. The duodenum of Wistar rats which were given PEDS showed significant difference with negative control in signal transduction activities of PKC $\mu$ , c-Raf & ERK 1/2 pathway ( $p < 0.001$ ).

**Conclusion:** PEDS contains the highest  $\beta$ -conglycinin level, effects to CCK plasma level, and to CCK signal transduction pathway through activities of PKC $\mu$ , c-Raf and ERK 1/2 in enteroendocrine cells in duodenum Wistar rats

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## 1. Introduction

The prevalence of obesity is definitely increasing and is widely spread in all over the world (1). This condition should be overcome because obesity can cause many side effects. One of options solution is to discover good nutrition sources that can reduce body weight. Soybean (Glycine Max L. Merr) is a good source of protein. It has two major protein fractions,  $\beta$ -conglycinin (7S) and glycinin (11S) (2).  $\beta$ -conglycinin's function was known to suppress food intake, decreasing appetite (3) reducing body weight and this effect may be due to stimulating gastrointestinal hormones, cholecystokinin (CCK) release (4). CCK is a peptide hormone of the gastrointestinal system that can act as a short

term food intake inhibitor, has been demonstrated to reduce food intake in a dose-related manner in different species and experimental designs (5, 6, 7, 8).

Protein can stimulate CCK secretion in STC-1 cells duodenum through signal transduction of G Protein Coupling Receptor (GPCR) mechanisms (9). There are many possibilities in the pathway, such as Protein Kinase C (PKC), DiAcyl Glycerol (DAG), Protein Kinase (PKA), c-Raf and Extracellular Signal-Related Kinase (ERK) or Mitogen Activated Protein Kinase (MAPK) (9). However, the cellular mechanisms by which the  $\beta$ -conglycinin induces CCK secretion remain to be clarified.

There hasn't been many studies about Indonesian soybean variety in the relation to CCK

although there are many varieties of soybean in Indonesia. Two sorts of soybean which are chosen in this research are local soybean Wilis and Detam 1 variety. The Detam 1 variety which are a high quality of soybean then were prepared into tempeh since we knew that fermentation process can raised the active substance in soybean and made easier to digest (10). The aims of this study are to measure which kind of soybean extract contains the highest  $\beta$ -conglycinin level, the effects to CCK plasma level, and to signal transduction pathway through PKC $\mu$ , PKA, c-Raf and ERK 1/2 in enteroendocrine cells in duodenum of Wistar rats.

## 2. Materials and Methods

Two sorts of soybean which are chosen in this research are Wilis and Detam 1 variety. Wilis variety is an Indonesian local soybean which is commonly planted by farmers in Indramayu. This soybean has a yellow flesh seed with yellow skin seed. It contained about 39 % protein (percentage from dry weight) (11,12).

The soybean Detam 1 variety was planted in Balai Penelitian Kacang dan Umbi-umbian (Balitkabi) plantation in Malang. This variety developed from a cross of 9837 introduction variety breeding with Kawi variety. Cultivated Detam 1 variety produced 2,51 ton per acre, seeds were harvested in 84 days. This soybean has a yellow flesh seed but covered with hard black seed skin. It contained 41.82 % protein (percentage from dry weight), much higher than another variety (10, 12). This variety was approved as a high quality soybean by Minister of Agricultural decree no 240/Kpts/SR.120/3/2008 date March 6th 2008 (13).

Then Detam 1 variety of soybean were made into tempeh. After that the seed and tempeh from Detam 1 and Wilis variety were extracted using Panthee procedures. There are 3 sorts of extracts which were examined in this study:

1. Protein extract of Wilis Soybean seed
2. Protein extract of Detam 1 Soybean seed
3. Protein extract of Detam 1 Soybean tempeh

The  $\beta$ -conglycinin level were analysed in vitro using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and coomassie brilliant blue (CBB) staining (14, 15). The CCK plasma level were measured in vivo in male Wistar rats by ELISA method and the PKC $\mu$ , c-Raf and ERK in CCK signal transduction pathway were measured in vivo by Western blot method.

The results were acquired by dividing the densitometry value expression of  $\beta$ -conglycinin from each extract with the pan actin value as internal control. From these results, we can conclude which extract protein of soybean are responsible for the stimulation of CCK secretion in enteroendocrine in duodenum of Wistar rats. This experimental study to normal Wistar male Rats, has got ethical clearance from Ethical Committee Maranatha Christian University No 148/KEP FK UKM-RSI/V/2009.

### 2.1 Fermentation Soybean Procedures to Make Tempeh (10, 16).

Procedures in making Tempeh include 8 steps process, boiling, skin seed peeling, soaking, washing, steaming, giving the yeast, packaging and stewing. Detam 1 and Wilis soybean seed were weighed 500 grams each, and then boiled. The next process is peeling the skin seed. It is necessary to be done because the mycellium of the yeast is difficult to break through the epidermis which contains horny materials. Soaking is the next step after the soybean skin were peeled. In the soaking process there will be an acid condition. The soybean seed pieces (without skin) which has been soaked were washed until the seed is not sleek. Then the materials were steamed like we steamed rice until the seed were soft and wellcooked.

The next step is giving the yeast or tempeh inoculum *Rhizopus oligosporus* or *laru* to the soybean seed then the materials were cooled. *Laru's* dosage is 1 gram for 1 kilogram soybean seed. The soybean seed which were already mixed with *laru*, were packed in plastic bags. The good growth of yeast is in the temperature of 20 – 37°C. Then the packages of this materials were kept for 2 days in the room temperature with good airflow (10, 16). From 500 gram raw soybean seed we produced 650 gram tempeh.

### 2.2 Soybean and tempeh protein extraction (Panthee method modification) (17).

Weigh each material 10 grams. Ground it in cool water (20°C) Knifetec 1,095 Sample mill for 20s. This setting produced soybean flour with relatively uniform particle size. Soluble protein was extracted for 1 h at room temperature while stirring from one gram of full fat soybean flour in a 1:15 (w/v) ratio with 0,2M Tris HCl buffer, pH 8,0 that contained 0,1 M  $\beta$ -mercaptoethanol. The mixture was centrifuged (10.000 X g) 10 min at

4°C. Upon removal of the fat layer, an aliquot (1 mL) of crude protein extract or supernatant was taken from each sample. Storage proteins and their polypeptides were dissociated in the crude extract by adding an equal volume of both 5% SDS and 0.1 M  $\beta$ -mercaptoethanol solution and warmed in a waterbath for 10 min (16).

### 2.3 SDS-PAGE and CBB staining (14).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (14). Protein concentrations were measured using a Bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with Bovine Serum Albumin (BSA) as a standard. It was carried out in 80- (high)  $\times$  100- (width)  $\times$  1-mm (thick) vertical slab gel; consisting of stacking gel mix, 5% total acrylamide, and main running gel mix, 20% acrylamide, prepared in 0.375M Tris-HCl, pH 8.8. Twenty micrograms of protein was run on a 5 - 20% Ready Gel (Bio-Rad, Tokyo). Electrophoresis was carried out at 100 v and 400 mA constant electric current for 100 min. Since there is no antibody specific for this protein extracts, protein bands were visualized by staining with 0.05% Coomassie brilliant blue R-250 (Bio-Rad, Tokyo) (14, 18,19).

Protein markers employed were myosin (206 kilo Dalton; kDa),  $\beta$ -galactosidase (118 kDa), bovine serum albumin (97 kDa), ovalbumin (54 kDa), carbonic anhydrase (37 kDa), soybean trypsin inhibitor (29 kDa), lysozyme (17 kDa) and aprotinin (6 kDa). Determination of expression levels of each molecule was conducted with pan-actin (Cell Signaling, MA) as a loading control. Comparison of these levels among each sample was performed by densitometer using acquisition into the Photoshop software (Adobe) and analyzed by the Quantity one (Bio-Rad) (18, 19).

The soy protein is composed of two major protein components,  $\beta$ -conglycinin (7S globulin of soybean) and glycinin (11S globulin of soybean).  $\beta$ -conglycinin is a trimeric protein composed of various combination of three subunits (namely,  $\alpha'$  - ~72 kDa,  $\alpha$  - ~68 kDa and  $\beta$ - ~52 kDa) and glycinin (acidic and basic subunits of ~35 kDa and ~20 kDa, respectively) (Thanh, 1977, Nielsen, 1985).  $\beta$ -conglycinin sub unit  $\beta$  51-63 sequence, has the most antiobesity effect by reducing appetite through stimulation secretion of Cholecystokinin (CCK) (20).

### 2.4 Diets and Animal Experimental Protocol

As many as 24 male Wistar rats (5-6 weeks), weight 145 - 183 gram, each were put in a cage in Farmacology laboratory Padjadjaran University Faculty of medicine and Hasan Sadikin Hospital Centre Bandung. The rats were given standard soyfree pellet feed and water ad libitum. After 7 days adaptation, they were divided randomly into 4 experimental groups of 6 rats, then were given for 14 days:

1. Protein Extract of Wilis Soybean seed (PEWS) dosage 20 mg/rats/d (n=6)
2. Protein extract of Detam 1 Soybean seed (PEDS) dosage 20 mg/rats/d (n=6)
3. Protein extract of Detam 1 Soybean tempeh (PEDT) dosage 20 mg/rats/d (n=6)
4. Negative control (Aquadest) (n=6) 1 ml/d

Cholecystokinin plasma level were measured from blood of the vein tail on the first day before treatment and 14th day after treatment using ELISA method (Phoenix Pharmaceutical. Cat. # EK-069-04 CCK (26-33) (Non sulfated), performed 45 minutes after rats were given treatment.

### 2.5 Prosedures to Measure (CCK) Plasma Level in Rats using ELISA Method (21)

On the first and on the 14th day after fasted for 12 hours, cholecystokinin plasma level each rats in all groups were measured by taking blood sample from vein tail blood 1 cc, then were put in a lavender vacutainer (# VT-6450) tube which contain 2 mg EDTA. Then the tubes were shaken and made like eight number movement to make a well mix plasma. Then they were centrifuged 1600xg for 15 mnts at 4°C. Five tubes were prepared to make a standard solution. Then they were added 50 $\mu$ l/well of standard, sample, or positive control, 25 $\mu$ l primary antibody and 25 $\mu$ l biotinylated peptide. Incubated at room temperature (20-23°C) for 2 hours. Then the immunoplate were wash 4 times with 350 $\mu$ l/well of 1x assay buffer. Added 100 $\mu$ l/well of SA-HRP solution. Then they were incubated at room temperature (20-23°C) for 1 hour. The immunoplate were washed 4 times with 350 $\mu$ l/well of 1x assay buffer then were added 100 $\mu$ l/well of TMB substrate solution. Incubated at room temperature (20-23°C) for 1 hour. Reaction were terminated with 100 $\mu$ l/well of 2N HCl. The absorbance O.D were read at 450 nm and calculated. The results were counted from two replications (duplo) and were analysed statistically using t-test paired.

## 2.6 Procedures to exam the signal transduction pathway through PKC $\mu$ , c-Raf and ERK in enteroendocrine cells in duodenum Wistar rats using Western Blot (22, 23)

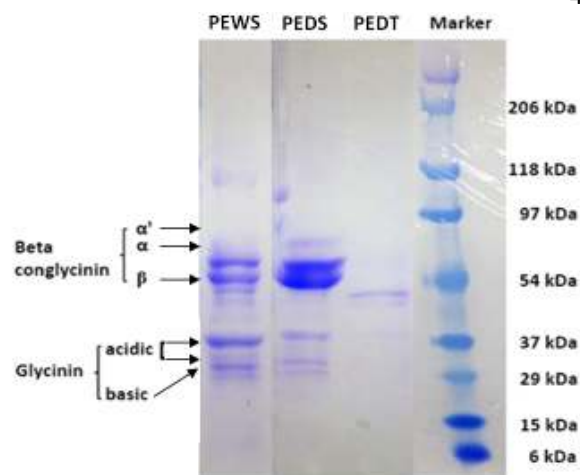
After overnight fasted, at the last day after 14 days of treatment, the rats were given the last soybean protein extracts dose of 20 mg via sondage. Then 45 minutes later, after the peak duration of action of Cholecystokinin, all the rats were sacrificed. The rat duodenum were cut about 10 cm, from the region of the ampulla Vater. Then the duodenum were prepared for the next step, to exam the signal transduction pathway through PKC $\mu$ , c-Raf and ERK using Western blot method. Stages conducted in Western blotting are as follows:

- 1). Intake of protein from the tissue by lysis proteins.
- 2). Calculation of protein level.
- 3). computerized level of protein from the plate reader.
- 4). Electrophoresis.
- 5). Transferring.
- 6). Blocking.
- 7). Giving antibodies:
  - PKA Antibody Thr 197 (Cell Signaling, MA) # 4781
  - PKC  $\mu$  Antibody or PKD Ser 744/748 (Cell Signaling, MA) #2054
  - c-Raf Antibody Ser 338 (Cell Signaling, MA) #9422
  - ERK Antibody Thr 202/204 (Cell Signaling, MA) # 9102
  - Pan-actin Antibody (internal control) 42 kDa (Cell Signaling, MA) #4968
- 8). Detection.
- 9). Stripping.

The difference of densitometry results of Western blotting bands of PKA, PKC $\mu$ , c-Raf and ERK 1/2 is the increase in PKA, PKC $\mu$ , c-Raf and ERK 1/2 activity in the signal transduction pathway of CCK in duodenum of rats after treated with PEWS, PEDS, and PEDT of 20 mg compared with untreated mice (only 1 ml distilled water/aquadest) by analysed of Western blot bands using acquisition into the Photoshop software (Adobe) and analyzed by the Quantity one (Bio-Rad). The results were read three times (triplo) by Scion densitometer and were analysed statistically using Analysis of Variance (ANOVA) continued with Duncan post Hoc test,

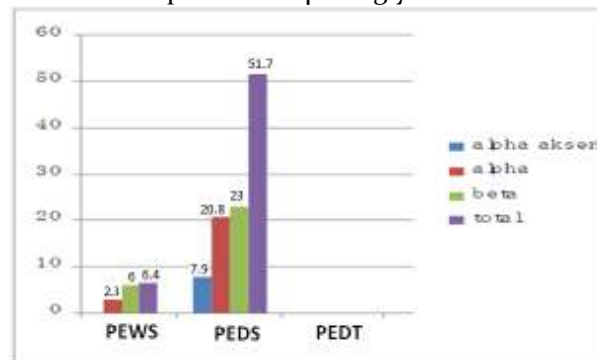
## 3. Result

The SDS-PAGE showed results as seen below:



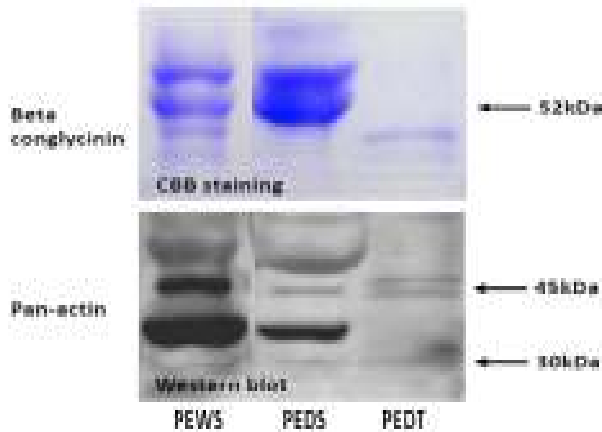
**Figure 1.** Polyacrylamide gel electrophoresis (SDS-PAGE) of  $\beta$ -conglycinin and glycinin from Indonesian soybean determined by pan-actin (Cell Signaling, MA). M. Molecular mass standards; Line # 1. Protein extract of Wilis Soybean seed (PEWS) Panthee Method Line # 2. Protein extract of Detam 1 Soybean seed (PEDS) Panthee Method Line # 3. Protein extract of Detam 1 Soybean tempeh (PEDT) Panthee Method

The bands of pan actin as internal control (Figure 3) were not equal probably due to the differences of either the purity or quality the extracts. However there was able to determine the expression of  $\beta$ -conglycinin from the extracts by dividing the densitometry value with the pan actin value as internal control. PEDT using Panthee modification method (Figure 2; line # 2) had the highest level of total  $\beta$ -conglycinin (51.7 densitometric unit, DU), alpha aksen subunit (7.9 DU) and alpha subunit (20.8 DU) also. On the other hand, PEWS Panthee method (Figure 2; line # 1) showed low concentrations of total  $\beta$ -conglycinin, 6.4 and 1.2 DU, respectively. However, PEDT Panthee method (Figure 2; line 3) showed no expression of  $\beta$ -conglycinin.



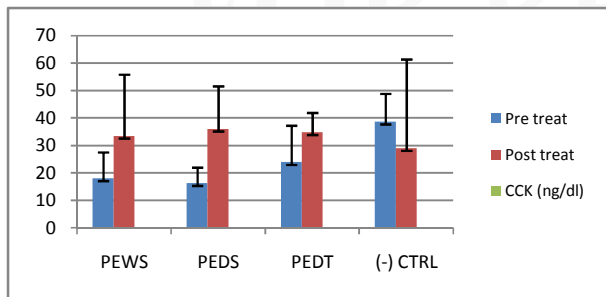
**Figure 2.** SDS PAGE Profile of Indonesian Soybean: Beta Conglycinin and Glycinin. Results of horizontal scanning densitometry performed by acquisition into Adobe Photoshop (Apple, Inc., Cupertino, CA) and analysis by the Quantity One (BioRad). The CRR staining showed results as seen below.





**Figure 3.** Coomassie brilliant blue (CBB) staining and Western blot analysis of  $\beta$ -conglycinin and its unit calculated as the arbitrary densitometric of bands after normalization of each expression level with pan-actin

After 14 days of treatment, there was tendency increasing CCK plasma level in all of treatment groups PEWS, PEDS, and PEDT although the results are non significant statistically ( $p > 0.05$ ). The PEDS group reached the highest level of CCK [pre 16.23 ng/ml (SD 5.69), post 36.03 ng/ml (SD 15.43) increased 19.80 ng/ml] (Table 2).



**Figure 4.** The measurement of Cholecystikinin (CCK) plasma level

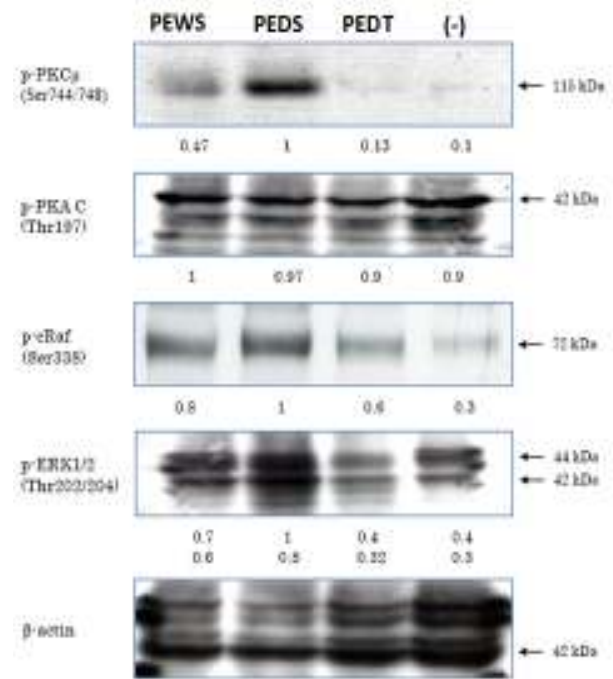
**Table 2.** Statistical Analysis Cholecystikinin (CCK) Plasma Level Pre and Post Soybean Extracts Treatment using t-test paired

Group Treatment	Measurement pre (ng/dl)	Measurement post (ng/dl)	Increased (ng/dl)	t test paired	p Value
PEWS	17.9667 (9.452)	33.5000 (22.250)	15.533	-1.221	0.347
PEDS	16.2333 (5.687)	36.0333 (15.429)	19.800	-1.846	0.206
PEDT	23.8667 (13.261)	34.8000 (7.002)	10.933	-3.024	0.094
(-) CTRL	38.6000 (10.153)	29.0000 (32.290)	-9.6	0.544	0.641
F (ANOVA)	0.876	0.105			
p Value	0.580	1.000			

p Value  $< 0.05 \rightarrow$  Significant (\*)

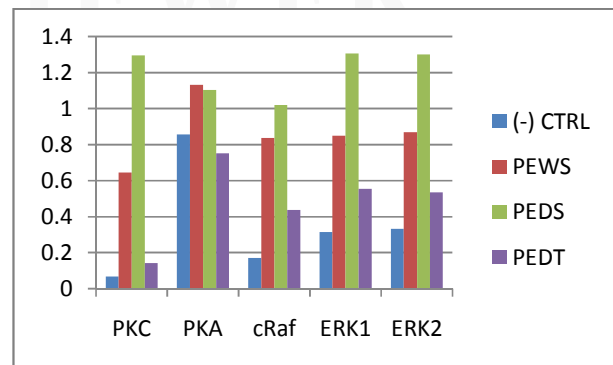
p Value  $< 0.01 \rightarrow$  Highly significant (\*\*)

The activities of PKC $\mu$ , c-Raf and ERK in enteroendocrine cells in duodenum of Wistar rats using Western blot showed results as seen below:



**Figure 5.** Duodenum Wistar rats with and without treatments, Western blot method

After the bands were read three times (triplo) and calculated using Scion Image densitometer, the average results showed as seen below:



**Figure 6.** Average activity of PKC PKA, cRaf, ERK1, ERK2 signal transduction of 4 groups of treatment

After analysed using ANOVA, the average results of PKA, PKC $\mu$ , c-Raf dan ERK 1/2 activities showed signigancny level (0.000) it means minimal one group were different signigantly. We concluded that the treatment respon signigantly different from the negative control. So we continue analysing using Duncan Post Hoc Test. And the Duncan test results PEDS showed the highest activities in PKC $\mu$ , c Raf, ERK1 and ERK2, but not in PKA pathway (Figure 6).

#### 4. Discussion

Total  $\beta$ -conglycinin level in PEDS was higher than in PEWS. Possibly, because the total protein level of Detam 1 Soybean seed (41.82 %) was higher than protein of Wilis soybean variety (39.00%) or its fermentation products (25.35%) (12). The seed (raw) of soybean without preparation is suggested to get the highest total  $\beta$ -conglycinin level, compared with the fermented or the soybean skin (24). Fermented process may decreased the protein content in the soybean. For illustration, at average dry raw soybean seed contained 34.9% of protein, but pure soybean tempeh only contained 18.3% (12). There was no  $\beta$ -conglycinin level in PEDT. So fermented soybean is not a good sources for  $\beta$ -conglycinin.

In response to CCK plasma level, there were increasing CCK plasma level in all group of treatments. After the data were analyzed with t-test paired, there were no significant difference between pre and post treatment. PEDS, which has the highest level of  $\beta$ -conglycinin can raise the CCK level 19.800 ng/dL (Tabel 2) but showed no significant result statistically  $p=0.206$ . It is clear that the higher  $\beta$ -conglycinin level stimulating the CCK plasma level higher. In another study, PEDS showed highly significant reducing body weight effect in Wistar rats ( $p=0.01$ ) (25,26). Cholecystokinin has been proved decreasing food intake in rats and pigs (5,6,7,8). Why the result are not significant, there were many possibilities for this reason and limitations in this study. The pre treatment CCK plasma level were measured from blood taken from the vein tails of the rats. While CCK plasma level post treatment were measured from blood which were taken from intra cardial of the rats just before they were sacrificed, because we need a lot of blood for another measurement. It may gave slight different results.

In CCK signal transduction, it has already known that peptone or protein hydrolysate can stimulate the expression, transcription and secretion of CCK via G Protein Coupling Receptor (GPCR) pathway in STC-1 duodenum cells (9). In his study, Hira stated that  $\beta$ -conglycinin peptone induced CCK secretion depends on Galphaq-mediated pathways in enteroendocrine cells (27). While Hara found that  $\beta$ -conglycinin can induce signal of calcium, and concluded that induced the CCK secretion through PKC pathway (28). The downregulation of this pathway still need to be investigated. In this study, we try to make the pathway more complete. Our study showed the down pathway are: PKC $\mu$ , c-Raf and ERK 1/2,

which proved through PEDS treatment, that contains high  $\beta$ -conglycinin level.

#### 5. Conclusion

Protein Extract of Detam 1 Soybean seed contains the highest  $\beta$ -conglycinin level, effects to CCK plasma level, and to CCK signal transduction pathway through activities of PKC $\mu$ , c-Raf and ERK 1/2 in enteroendocrine cells in duodenum of Wistar rats.

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#### Conflict of Interest

The authors report no conflicts of interest

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