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Peptides Derived from Dolicholin, a Phaseolin-like Protein in Country Beans (*Dolichos lablab*), Potently Stimulate Cholecystokinin Secretion from Enteroendocrine STC-1 Cells

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Peptides derived from soybean β-conglycinin and pork protein stimulate cholecystokinin (CCK) secretion from the enteroendocrine cells (EECs) and suppress food intake. Here we examined CCK-releasing activities from the enteroendocrine cell line STC-1, in peptides derived from underutilized legumes, and found much higher activity in the peptic hydrolysate of Country beans (CBP) compared to that from other legume-derived peptides including β-conglycinin peptone. Active components in CBP were separated into acetonitrile-soluble fractions, but the activities were abolished after pronase treatment. To identify the Country bean protein containing the active peptides, Country bean protein extracts in an alkaline solution (CBE) were fractionated based on isoelectric point or molecular weight. Peptones prepared from CBE fractions containing a 51 kDa major protein stimulated CCK release, but other fractions did not. N-Terminal sequence analysis indicated that the 51 kDa protein is a phaseolin-like globular protein, and we designated this protein dolicholin. These results indicate that Country bean-derived peptides are very potent legume peptides in stimulating CCK secretion from EECs and that the stimulant peptides originate from dolicholin, a newly identified phaseolin-like globular protein in Country beans.

KEYWORDS: ***Dolichos lablab*; legumes; cholecystokinin; peptone; enteroendocrine STC-1 cells**

INTRODUCTION

The search for new food proteins or derivative peptides has become of increasing interest due to recent reports indicating that some specific peptide structures derived from parent food proteins are biologically active with a variety of health benefits. These bioactive peptides may lead to the development of many functional food components (*1–3*). The prevalence of lifestyle-related diseases such as obesity and diabetes has become an increasing problem around the world. Thus, the development of such functional food components may provide an opportunity for the prevention of these lifestyle-related diseases, particularly by reducing their risks through improved dietary practices in daily life. In this regard, the antihypertensive, hypocholesterolemic, immunomodulatory, opioid, antioxidant, and appetite-suppressive activities of many protein hydrolysates or peptides have already been determined (*4–8*).

The appetite-suppressive peptides or their parent protein hydrolysates induce satiety by stimulating cholecystokinin (CCK) secretion, a gut-brain satiety hormone from the proximal small intestine. CCK is secreted from the enteroendocrine I cells in the lining of intestinal epithelium upon ingestion of nutrients (*9–11*). It plays a major role in stimulating pancreatic enzyme secretion, gallbladder contraction, inhibition of gastric emptying, and appetite suppression. In rats, an elevation in CCK secretion has been shown to induce satiety after the gastric or duodenal delivery of peptones (*12, 13*). The sensory mechanisms by which these nutrients stimulate the EECs are still unclear, and it is believed that dietary protein-mediated CCK release is regulated by endogenous, trypsin-sensitive CCK-releasing peptides in the luminal protease-mediated feed-back mechanism (*14*). However, some in vivo and in vitro studies, including ours, found that dietary protein and its peptide act directly on CCK-producing EECs to stimulate CCK release independent of endogenous CCK-releasing peptides (*15–17*). The later investigations were further supported by the findings that peptones (protein hydrolysates) stimulated CCK secretion in a CCK-producing murine enteroendocrine cell line, STC-1 (*18–20*).

Recently, we identified peptones from soybean β-conglycinin and pork protein that stimulate CCK release with appetite-suppressive effects and later identified the satiety fragment from β-conglycinin (*4, 17, 20*). Other researchers identified the hypocholesterolemic peptide from soybean protein (*21*). There has been a world-wide trend to search for unconventional

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legumes as potential peptide sources for use as functional food components or nutritional supplements (*22–25*). Unlike soybeans and a few other well-known legumes, many unconventional legumes remain underutilized. The potential of some underutilized legumes from developing countries as functional food components has been reported (*26*); however, the effects of lesser known legume proteins and peptides on CCK release have not been studied.

The aim of the present study was to search for new CCK-releasing dietary peptides or their parent proteins from underutilized legumes. Here we investigated two underexploited legumes, Country beans (*Dolichos lablab*) and Yard long beans (*Vigna sesquipedalis*) and compared their CCK-releasing efficacy with that of soybean β-conglycinin in the enteroendocrine cell line, STC-1, which has already been characterized as a suitable model for the study of CCK release (*18–20, 27–29*).

MATERIALS AND METHODS

Materials. Country bean (CB, East West Seed Bangladesh Ltd.) and Yard long bean (YLB, Local cultivar) seeds were obtained from local markets in Bangladesh and cleaned by manual sorting to remove extraneous materials. Purified soybean β-conglycinin flour was a gift from Fuji oil Co., Ltd. (Osaka, Japan). Seed specimens were kept in our laboratory herbarium for future reference. All chemicals were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan, unless otherwise noted.

Preparation of Legume Protein Hydrolysates. Country bean and Yard long bean seeds immersed in H_2O were heated at 100 °C for 30 min to soften the husks, which were then removed using a scalpel. Beans (10 g each) were homogenized in water (200 mL) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland), adjusted to pH 7.0, boiled (100 °C) for 60 min, centrifuged at 3750*g* to get supernatant, and then lyophilized to produce a Country bean and Yard long bean boiled water extract (CBW and YLBW, respectively). Pepsin hydrolysis was performed as previously described with slight modifications (*4*). Briefly, Country bean and Yard long bean seeds immersed in H_2O were heated for 30 min, and the husks were removed. These beans together with soybean β-conglycinin flour (10 g each) were suspended in phosphate buffer (200 mL) and homogenized as described above. They were then treated with pepsin (10 870 units/g of substrate, Sigma Aldrich, St. Louis, MO) at pH 1.8 and 37 °C for 10 min followed by immediate boiling, neutralization, and desalting with $Ca(OH)_2$, centrifugation at 3750*g*, and lyophilization of the supernatant. The peptones obtained are labeled as Country bean peptone (CBP), Yard long bean peptone (YLBP), and β-conglycinin peptone (BconP), respectively. CBP was separated into soluble and insoluble fractions by the addition of cold acetonitrile (ACN) up to 50% (final concentration) on ice for more than 30 min. The resulting supernatant and precipitate were separated following centrifugation at 1050*g* and 4 °C for 15 min and freeze-dried as the CBP-ACN soluble fraction (CBP-AS) and CBP-ACN insoluble fraction (CBP-AI).

For pronase treatment, dissolved CBP or CBP-AS (100 mg in 20 mL, pH 7.0) was treated with the protease pronase at 225 PUK (proteolytic unit/g of substrate, Pronase Protease, *Streptomyces griseus*, Calbiochem, EMD Biosciences Inc., La Jolla, CA) for 60 min at 37 °C. The reaction was stopped by boiling, and the pronase-treated CBP or CBP-AS was freeze-dried after readjustment to pH 7.0.

Isoelectric Point (pI) Fractionation of Country Beans Protein Extract (CBE). Country bean seeds were immersed in H_2O at 4 °C and the husks removed and then the seeds were homogenized in H_2O (30 g/300 mL) and the pH was adjusted to 9.0 using 0.1–1 M NaOH (as appropriate). The homogenate was stirred for 2 h at 4 °C and centrifuged at 3750*g* for 15 min. The resulting supernatant (Country beans protein extract, CBE) was filtrated (0.2 μm) and immediately subjected to isoelectric point (pI) fractionation. First, CBE (250 mL) was adjusted to pH 7.0 (0.1–1 M HCl) and stirred for 2 h at 4 °C, and precipitate was collected with centrifugation at 850*g*, 4 °C for 15 min, extensively washed twice with water at the same pH and then centrifuged at a higher speed (9000*g*, 4 °C, 5 min). The resulting precipitate was freeze-dried as the pI fraction 1 (F1). This procedure was repeated with the pH of the remaining supernatants adjusted to 6.0, 5.0, or 4.0 during the fractionation step followed by collection of the resulting precipitates as F2 (pH 6.0), F3 (pH 5.0), F4 (pH 4.0), respectively. Ethanol was added to the remaining supernatant (final concentration of 80%) and the sample was kept on cold ice for more than 30 min to facilitate protein precipitation. The resulting precipitate was freeze-dried as F5 (EtI), and the remaining supernatant was dried on a rotary evaporator as F6 (EtS). The fractions obtained were analyzed by SDS–PAGE. The fractions (F2–F6, except for F1 due to a very low yield) were treated with pepsin as described above. The peptones produced from each protein fractions are labeled as F2P, F3P, F4P, F5P, and F6P. The protein and peptide content of the protein fractions and peptones were determined by the method of Lowry (*30*) using bovine serum albumin (BSA) as a standard and expressed as the protein or peptide purity (%) against BSA. Average molecular weights of the peptones were determined by FPLC (Superdex Peptide 10/300 GL column, Amersham Pharmacia). The CCK-releasing activities of these peptones were studied to identify the active protein fraction(s).

Molecular Weight (MW) Fractionation of Country Beans Protein Extract (CBE). Fifty (50) mg of CBE was loaded onto a HiLoad 16/60 Superdex 200 column (Amersham Pharmacia) connected to a fast protein liquid chromatography (FPLC) system (AKTA explorer 10S, Amersham Pharmacia) with 50 mM phosphate buffer (pH 7.2) as the running buffer. Flow rate was controlled at 1.0 mL/min. The procedure was performed repeatedly in isocratic mode with fixed volume fractionations (5 mL each). The fractions were combined to produce several peak fractions as FA, FB, FC, FD, and FE. After membrane dialysis to remove salts, the fractions were analyzed by electrophoresis to determine MW distributions. The fractions FA, FB, FC, FD (except for FE due to a very low yield), and CBE were treated with pepsin as described above to produce peptones (FAP, FBP, FCP, FDP, and CBEP, respectively). They were studied for CCK-releasing activities to identify the active protein fraction(s).

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Protein fractions were run on 15% discontinuous denaturing gels (*31*) either at 12.5 mA for 4.5 h in a Hoefer SE 600/SE 660 electrophoresis apparatus (Amersham Biosciences Corp., San Francisco, CA) or at 100 V for 40 min in a Mini-PROTEAN 3 Cell electrophoresis apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) followed by staining with 0.25% Coomassie blue G250 solution (Bio-Rad Laboratories, Inc., Hercules, CA). Precision Plus Protein Standards (Bio-Rad Laboratories, Inc., Hercules, CA) were used as molecular weight markers, and the molecular weights of Country bean major proteins were determined calculating electrophoretic mobility of these standard protein markers.

N-Terminal Amino Acid Sequencing. CBE was run on SDS–PAGE in the presence of β-mercaptoethanol, and the separated protein bands were transferred onto a PVDF membrane (Immun-Blot PVDF Membrane, Bio-Rad Laboratories, Inc., Hercules, CA) by the wet blotting method (4 h at 50 V). The N-terminal amino acid sequence of the Country bean major protein band stained by CBB (0.25% Coomassie G250) was determined using a Procise 492 (Applied Biosystems Inc.) protein sequencer. The N-terminal amino acid sequence of the major Country bean protein was compared with known sequences using the BLAST database (*32*).

Cell Culture and Study for CCK-Releasing Activities. STC-1 cells were kindly provided by Dr. D. Hanahan (University of California, San Francisco, CA). These cells were originally derived from an intestinal endocrine tumor obtained from double transgenic mice (*33*). STC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L glucose, with L-glutamin, without sodium pyruvate; GIBCO BRL 12100-038, Grand Island, NY) containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin under a humidified 5% CO_2 atmosphere maintained at 37 °C. Cells between passage 30 and 40 were used at 80–90% confluence.

For secretion studies, 1.25×10^5 cells were seeded into 48-well plates and used when they reached subconfluence after culturing for 2–3 days. The cultured cells were then washed twice with HEPES buffer (140 mM NaCl, 4.5 mM KCl, 20 mM Hepes, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, pH 7.4), and test hydrolysates were dissolved

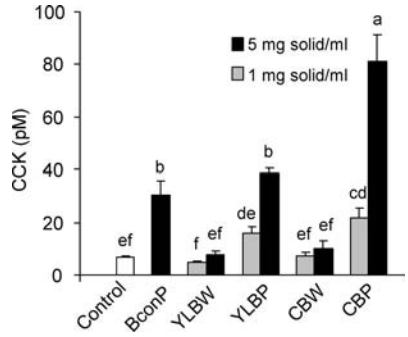

Figure 1. CCK-releasing activity after exposure to β-conglycinin peptone (BconP, a positive control), Yard long bean peptone (YLBP), Country bean peptone (CBP), Yard long bean boiled water extracts (YLBW), and Country bean boiled water extracts (CBW) for 60 min at 1 and 5 mg of solid/mL, the Control contained no peptide. Values are means ± SEM of three repeated measurements. Means without a common letter are significantly different ($P < 0.05$).

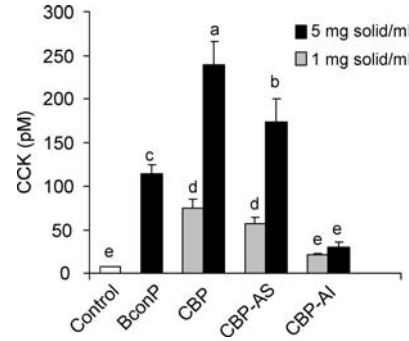

Figure 2. CCK-releasing activity after exposure to β-conglycinin peptone (BconP, a positive control), Country bean peptone (CBP), Country bean peptone acetonitrile soluble fraction (CBP-AS), and Country bean peptone acetonitrile insoluble fraction (CBP-AI) for 60 min at 1 and 5 mg of solid/mL; the Control contained no peptide. Values are means ± SEM of three repeated measurements. Means without a common letter are significantly different ($P < 0.05$).

in HEPES buffer (1–5 mg/mL solid or protein weight) were added to the wells. HEPES buffer without hydrolysate was added as negative control (control). After incubation for 60 min at 37 °C in the CO_2 incubator, the medium was collected on ice, centrifuged at 850*g* at 4 °C for 5 min to remove cells, and the supernatant was stored at −50 °C until CCK measurement. The released CCK was measured by a commercially available enzyme immunoassay kit (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA).

Calculations and Statistical Analysis. Results of the CCK secretion study are expressed as concentrations of CCK (pM) in the supernatant after incubation with or without stimulants or expressed as relative values of CCK release. All results of CCK secretion study are expressed as means ± SEM and analyzed with one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$). The molecular weight of Country bean major protein in SDS–PAGE was determined by plotting a log of molecular weights versus electrophoretic mobility of the standard protein markers. The average molecular weights of peptones were determined constructing a calibration curve using known molecular weight standards versus their retention times in FPLC and calculated from the total resolution area obtained for these peptones within their retention times.

RESULTS AND DISCUSSION

Effects of Legume Protein Hydrolysates on CCK Release. The aim of the present study was to search two underutilized legumes for new CCK-releasing dietary peptides and to identify the parent proteins. Peptones prepared from the three legumes tested stimulated CCK secretion, with CBP having the highest activity in a dose-dependent manner (**Figure 1**). On the other hand, the boiled water extracts (CBW and YLBW) of the two legumes had no effect. YLBP produced a similar effect, while CBP produced a higher stimulation than did BconP, which has already been reported as a potent CCK-releasing and appetite-suppressing peptide. CBP at 5 mg/mL increased CCK secretion 2.5-fold higher than did BconP. Previously, we identified specific peptide structures in BconP involved in the induction of satiety through CCK release (*4*). Our results from the present study suggest that Country bean contains a protein with putative peptide structure(s) possessing higher biological activities than either BconP or YLBP and is able to induce CCK release from EECs.

This initial screening provided us with the basis for further study on the characterization of Country beans. The active components in CBP are thought to be small or mid-size peptides as the ACN soluble fraction (CBP-AS) but not the ACN insoluble fraction (CBP-AI) stimulated CCK secretion (**Figure 2**). The putative active peptides in CBP are separated into CBP-AS; however, still a higher CCK-releasing activity is observed for CBP than for CBP-AS. Possibly, the ACN treatment denatured some of active peptides in CBP. CBP and CBP-AS were treated with pronase to destroy peptide structures. Pronase is a nonspecific potent protease that can digest proteins or peptides into single amino acids. The results that both CBP and CBP-AS pretreated with pronase abolished the CCK-releasing activity from EECs confirmed that the activity originates from peptide(s) rather than in free amino acids or other components included in CBP (**Figure 3**). This is the first report showing that peptides derived from an underutilized legume Country bean protein potently stimulate CCK secretion from EECs.

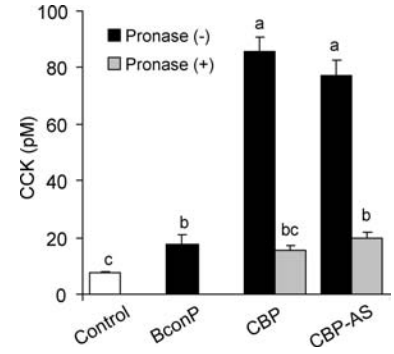

Figure 3. CCK-releasing activity after exposure to β-conglycinin peptone (BconP, a positive control), Country bean peptone (CBP), and Country bean peptone acetonitrile soluble fraction (CBP-AS) with or without pronase treatment, for 60 min at 5 mg of solid/mL; the Control contained no peptide. Values are means ± SEM of three repeated measurements. Means without a common letter are significantly different ($P < 0.05$).

Identification of Country Bean Proteins for Active Peptides using pI Fractionation. The yields and protein contents of Country bean proteins with different pI fractions are shown in **Table 1**. The CBE solid yield was around 15%, and its protein content was ~72%, which are comparable with those of Country bean crude extracts in previous reports (*22*, *34*). CBE pI fractions were treated with pepsin to identify the parent proteins of the active peptides responsible for CCK release (excluding F1 due to a very low yield). The peptide contents and average molecular weights of those peptones are also shown in **Table 1**. The peptide fractions were exposed to the cells with the proportion of solid yield in pI fractionation (**Figure 4A**) or with the same peptide concentrations (**Figure 4**B) as determined by the Lowry's protein assay (**Table 1**), to evaluate the contribution

Table 1. Summary of pI Fractionation Yield, Protein Content of the Fractions, and Peptide Content of the Prepared Peptones with Their Average Molecular Weights[a]

	solid yield (%)	protein content (%) of fractionated proteins[b]	labels of peptones from each fraction	peptide content (%) of peptones[b]	average molecular weights (Da) of peptones[c]
Country beans			CBP[d]	60.7	2909.3
CBE[e]	14.7	71.9	CBEP[f]	65.4	2802.4
F1 (pH 7.0)[g]	0.1				
F2 (pH 6.0)[g]	3.9	80.1	F2P[h]	74.4	3826.6
F3 (pH 5.0)[g]	46.3	93.0	F3P[h]	91.9	3901.7
F4 (pH 4.0)[g]	1.7	37.6	F4P[h]	65.9	3281.4
F5 (EtI)[g]	10.2	58.1	F5P[h]	80.0	3815.0
F6 (EtS)[g]	37.9	4.2	F6P[h]	6.4	257.7

[a] Details have been described in Materials and Methods. [b] Lowry protein assay (*30*). [c] Determined by FPLC. [d] Country bean peptone (CBP) produced directly from Country beans. [e] Country bean protein extract (CBE) extracted from Country beans. [f] Country bean protein extract's peptone (CBEP) produced from CBE. [g] F1-F6 (pI protein fractions produced from CBE). [h] F2P-F6P (respective peptones produced from pI protein fractions).

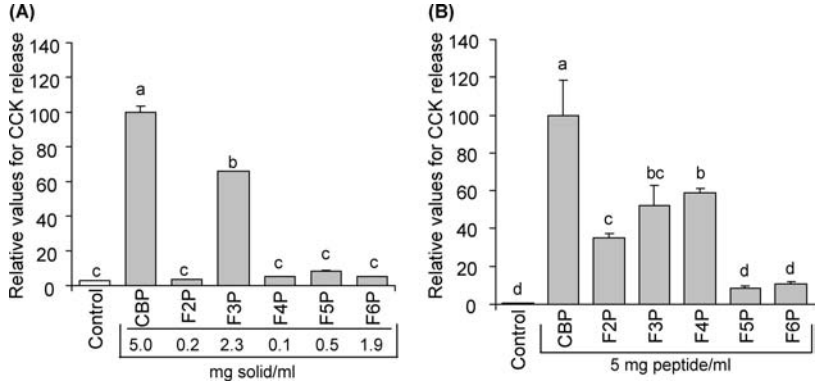

Figure 4. CCK-releasing activity after exposure to Country bean peptone (CBP) and peptones of Country bean protein extracts (CBE) isoelectric point (pI) fraction F2, F3, F4, F5, and F6 (F2P, F3P, F4P, F5P, and F6P, respectively; shown in Table 1) for 60 min. (**A**) CBP and fraction peptones were exposed as a proportion of their solid yield in pI fractionation. Numbers below the respective bars denote peptide–solid amounts exposed/mL except for the Control which contained no peptide. (**B**) CBP and fraction peptones were exposed at 5 mg of peptide/mL as determined by Lowry's protein assay, except for the Control which contained no peptide. Values are means ± SEM of three repeated measurements. Means without a common letter are significantly different ($P < 0.05$).

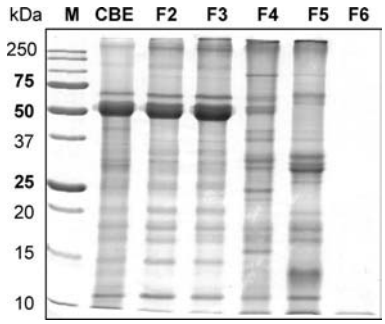

Figure 5. SDS–PAGE profile of Country bean protein extracts (CBE) and its different isoelectric point (pI) fractions: M (molecular weight markers), CBE, F2 (pH 6.0), F3 (pH 5.0), F4 (pH 4.0), F5 (EtI), F6 (EtS).

of each fraction to CBP activity. The CCK-releasing activities of these peptide fractions were compared as relative values for CBP. The peptone prepared from F3 (F3P) had the highest effect on CCK secretion when exposed, on both a proportion of yield and peptide concentration basis (**Figure 4 A**,**B**). Peptones derived from other fractions, however, failed to stimulate CCK secretion, except for those from the small yielding fractions F2 and F4 (F2P and F4P, respectively). F4P had a similar and F2P had a lower effect than F3P when exposed to the cells on a peptide concentration basis (**Figure 4B**). SDS–PAGE analysis identified a major protein in CBE as shown in **Figure 5**, which was calculated to be 51 kDa. Fraction 3 (F3), the highest yielding fraction both by weight and protein content, also contained a major 51 kDa protein identical to that in CBE (**Table 1**). These results suggest that the active peptides in Country bean responsible for the pronounced stimulation of CCK secretion originate from the major 51 kDa protein. Peptides derived from F2 (F2P) and F4 (F4P) also stimulated CCK secretion when exposed according to the peptide concentration basis as mentioned above. F2 also contained the 51 kDa protein, identical to that in F3, as a major protein. F4P stimulated CCK secretion to a similar degree as F3P, although F4 contained the 51 kDa protein as minor component, suggesting the existence of an active peptide derived from protein(s) other than the 51 kDa protein. However, F4 had a very low yield, and its contribution to the activity of whole CBE is very low.

Identification of Country Bean Proteins for Active Peptides by Molecular Weight (MW) Fractionation. The gel filtration profile of CBE and fixed volume fractions are presented in **Figure 6A**. The SDS–PAGE pattern (**Figure 6B**) shows unexpected results in that the major 51 kDa protein appeared in both FA and, more particularly, FB when characterized with SDS–PAGE. It is possible that FA contains an aggregated form of the 51 kDa FB protein and that FA also contains components of more than 250 kDa, which might complex forms of the 51 kDa protein with Country bean components other than proteins, for example, polysaccharides or polyphenols. Peptones prepared from all MW fractions and CBE (FAP, FBP, FCP, FDP, and CBEP, respectively) were applied to the cells to identify the active fractions (FE was excluded due to a very low yield) according to their fractionation areas in the gel filtration profile. Both FAP and FBP stimulated CCK secretion to a similar degree

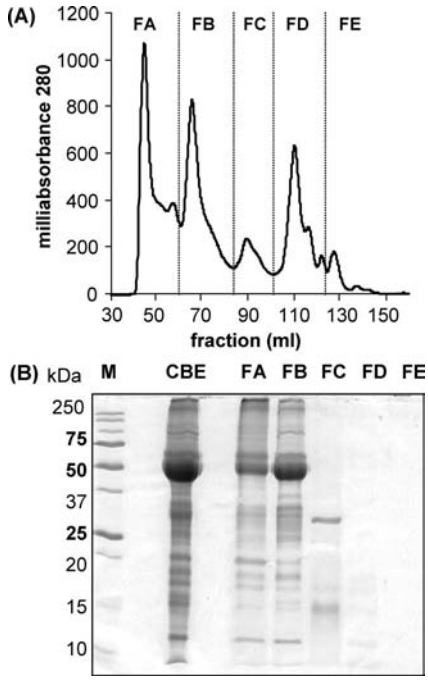

Figure 6. (**A**) Gel filtration profile of Country bean protein extracts (CBE) on a HiLoad 16/60 Superdex 200 column for molecular weight fractionation. Eluted fractions were combined as FA, FB, FC, FD, and FE as shown. (**B**) SDS–PAGE patterns of each fractions of CBE after FPLC-gel filtration using the HiLoad 16/60 Superdex 200 column.

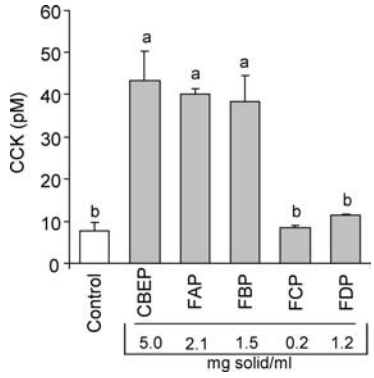

Figure 7. CCK-releasing activity after exposure to Country bean protein extract (CBE)'s peptone (CBEP) shown in Table 1 and peptones of CBE fractions FA, FB, FC, and FD shown in **Figure 6A** (FAP, FBP, FCP, and FDP, respectively) for 60 min. CBEP was exposed at 5 mg of solid/mL, while fraction peptones were exposed as a proportion of their molecular weight fractionation area in the gel filtration. Numbers below the respective bars denote peptide–solid amounts exposed/mL except for the Control which contained no peptide. Values are means ± SEM of three repeated measurements. Means without a common letter are significantly different ($P < 0.05$).

as CBEP, whereas FCP and FDP showed no activity (**Figure 7**). In the gel filtration profile, FA, FB, FC, and FD accounted for 42.4, 28.9, 4.4, and 24.3%, respectively, of the total resolution area. The results obtained from pI fractionation afford convincing evidence that the CCK-releasing peptides originate from the major 51 kDa protein in CBE. FAP showed a higher activity with a lower 51 kDa protein content, although FAP might include protein complexes with polysaccharides or polyphenolic compounds as mentioned above. Country bean seeds together with other legumes were reported to be a potential source of complex carbohydrate, dietary fiber, and phenolic compounds besides proteins (*35, 36*), and a study suggested carbohydrate moieties with proteins to be important for the peptide activity in CCK release (*15*). Future studies are needed to clarify whether the protein macrocomplexes in FA are involved in the enhancement of CCK release.

Table 2. Partial N-Terminal Amino Acid Sequence of Country Bean Major Protein and Sequence Analysis of Homology with Other Proteins

protein	N-terminal amino acid sequence	kDa	sequence analysis[a]
CBE major protein	IVH**QNNPFYFNSD**	51	90% identity to 9 aa (Q-NPFYFNSD) overlap with phaseolin α- and β-type precursors of *Phaseolous vulgaris*, 80% identity to 8 aa (Q-NPFYF-SD) overlap with phaseolin precursors of *Phaseolous lunatus*, 72% identity to 8 aa (H-N-NPF-FNS) and 66% identity to 8 aa (-NNPFYF---N) overlap with α and β-chain precursor respectively of soybean β-conglycinin (*Glycine max*), 100% identity to 9 aa (NNPFYFNSD) overlap with adzuki bean (*Vigna angularis*) 7S globulin and mung bean (*Vigna radiata*) 8Sα globulin (*37, 38*)

[a] Using BLASTP 2.2.17 (*32*) unless otherwise stated.

N-Terminal Amino Acid Sequence of the 51 kDa Protein. The partial N-terminal amino acid sequence of the 51 kDa CBE major protein containing the active peptides showed significant homology (66–100%) with some legume seed storage proteins including soybean β-conglycinin (**Table 2**). The partially obtained sequence had a very strong homology with the phaseolin α-type and β-type precursors of *Phaseolus vulgaris* showing 100% identity to the last eight amino acid residues, and a 100% identity with nine amino acids in adzuki bean (*Vigna angularis*) 7S globulin (MW 54–56 kDa) and mung bean (*V. radiata*) 8S globulin (MW 49 kDa) (*37, 38*). The obtained sequence was most closely matched with phaseolin in various legumes in the NCBI protein–protein BLAST comparisons. Phaseolin has been described as the major legume seed storage 7S globular protein with heterogeneous trimeric polypeptides (*39*). Country bean globulin includes at least three proteins with a molecular weight of 51–64 kDa, accounting for 48% of the total seed proteins (*40*). Considering the size of the major band in SDS–PAGE analyses and available data from this study as well as other literature, the major protein in CBE appeared to be a phaseolin-like globular protein. We designated the Country bean protein responsible for occurring the active peptide as dolicholin from its Latin name *Dolichos lablab*. The Country bean major protein (dolicholin) is highly homologous with soybean β-conglycinin; however, the peptides derived from dolicholin stimulate CCK secretion to a greater degree than does BconP from EECs. The peptide structure(s) from dolicholin involved in the induction of CCK release is not yet known. However, it is possible that some common peptide structures in dolicholin and β-conglycinin may be involved in the activation of CCK secretion. Previously, we reported strong membrane binding associations of CCK-releasing appetite-suppressive peptides in in vivo studies and a correlation with intracellular calcium responses in cellular models, indicating the presence of unknown cell surface receptors that may convert extracellular signals into the intracellular signal transduction pathways (*17, 20, 41*). Recently, we found that the arginine-rich peptide sequence in soybean β-conglycinin stimulated CCK secretion through binding with an unknown G-protein coupled receptor (GPCR) and other receptors via both Ca-dependent and

-independent intracellular signaling pathways (*4*, *42*). The dolicholin derived peptides and their stimulatory mechanisms at cellular levels must be clarified in future studies.

In summary, we have identified a new phaseolin-like protein in Country beans, designated dolicholin, and found that the dolicholin-derived peptides stimulate CCK secretion more potently than do known soybean β-conglycinin peptides from EECs. The complete protein sequence of dolicholin must now be identified for the efficient utilization of this underexploited legume.

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