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Full Length Research Paper

Purification and characterization of novel truncated fragments of bioactive proteins from porcine intestine with effects on insulin secretion

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During purification of porcine gut polypeptides with respect to glucose-induced insulin secretion from pancreatic β cells, two novel fragments of the known gastrointestinal peptides were isolated and chemically characterized. They are truncated forms of phosphatidylethanolamine-binding protein ($PEBP_{93-124}$) and valosin ($Valosin_{3-25}$). These novel fragments, $Valosin_{3-25}$ and $PEBP_{93-124}$, showed stimulatory activities on glucose-induced insulin secretion from pancreatic β cells. That the novel fragments had their respective bioactivities imply that these novel peptide-fragments could be the novel processed peptide-forms in organism. Therefore, isolation and chemical characterization of bioactive peptides from natural materials in the post genomic and bio-informational era are still necessary and will help scientist to have deeper insight into proteomics. In addition, these purified porcine gut polypeptides may provide an option to the treatment of Diabetes and also bring limelight to the mechanism of Diabetes manifestation.

Key words: Insulin, truncated peptide, phosphatidylethanolanmine binding protein, valosin, glucose-dependent insulinotropic polypeptide.

INTRODUCTION

The achievements in Molecular Biology have revolutionized our understanding of life sciences and medicine sciences. The dramatic expansion of the knowledge has broken the classical point of one gene corresponding to one protein. DNA sequences of genes may only give the existence and blueprint of pro-proteins. To truly and perfectly understand "From DNA sequence to biological function" (Oliver, 1996), Molecular Biology is therefore very limited, which cannot reveal the essential details of protein biochemistry and the function structures of those proteins at gene level. When we isolated gut polypeptides that affect insulin release from rat pancreatic β cells, several novel fragments of known gastrointestinal proteins have been purified and chemically characterized. We found that the novel peptide fragments have specific bioactivities respectively. The biofunctions of the original corresponding proteins have been, or at least in part, clearly characterized, which show these proteins play crucial roles in organisms (Bonetto et al., 1995). The

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Abbreviations: CTIP, Concentrate of thermostable intestinal polypeptides; **TFA,** trifluoroacetic acid; **HFBA,** heptafluorobutyric acid; **GIP_{1-42},** 1 to 42 residues of gastric inhibitory polypeptide; **GIP_{1-39},** 1 to 39 residues of gastric inhibitory polypeptide; **$PEBP_{93-124}$,** 93 to 124 residues of phosphatidylethanolamine-binding protein; **$Valosin_{3-25}$,** 2 to 25 residues of Valosin.

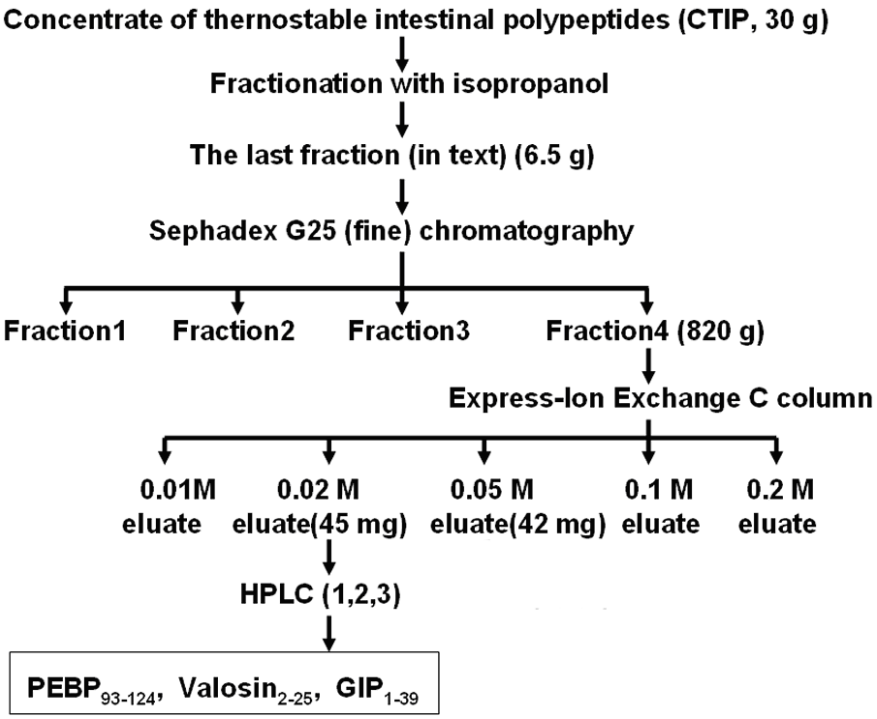

Figure 1. Preparation scheme of porcine fragments of gastrointesitinal polypeptides.

truncated peptide may have higher activity than its original peptide or different activities from its original peptide, such as we ever reported $GIP_{1–39}$ has higher activity than the original peptide GIP_{1-42} on insulin secretion (Xie et al., 2004).

We reported herein purification and characterization of two truncated fragments of the known bioactive proteins such as Phosphatidylethanolanmine Binding Protein (PEBP) and Valosin from porcine intestine, with stimulatory activities on glucose-induced insulin secretion from pancreatic β cells. Our results indicated that to isolate and chemically characterize proteins from the natural materials in the post genomic and bio-informational era are still necessary, which will help us to obtain more knowledge of protein processing and a deep insight into proteomics, and also, these purified may provide an option to treat Diabetes and bring a light to the mechanism of Diabetes.

MATERIALS AND METHODS

Peptide isolation

The full isolation procedures that referred our previous paper (Xie et al., 2004) were outlined in Figure 1. The CTIP 30 g was dissolved in 0.24 L water, then added 1.2 ml thiodiglycol to the mixture for antioxidation, followed by addition of 1.08 L isopropanol in the solution and stayed for 2 h at the room temperature. A precipitate was removed by centrifugation. An additional 1.32 L isopropanol that was precooled to -20°C was added into the supernatant. After 24 h at -20°C, a precipitate was removed with suction filtration. The filtrate was adjusted to pH 5.8-6.1 by 0.5 M potassium acetate in 90% ethanol adjusted to pH 8.0 with HCl. Staying for 48 h at -20°C, a white precipitate was collected by suction filtration and washed one time on the filter with isopropanol, and then with ether. The remaining ether was evaporated in vacuum.

6.5 g crude peptides powder was obtained and dissolved in 300 ml 0.2 M acetic acid. The peptide solution was chromatographied on a Sephadex G-25 (fine) column (10×90 cm) in 0.2 M acetic acid as showing in Figure 2. After lyophilization, Fraction 4 (820 mg) having a stimulatory effect on insulin secretion from rat β cells analyzed as described (Chen et al., 1988) was dissolved in 81.5 ml 0.01 M NH_4HCO_3 at pH 8.0, and stirred slightly for 20 min. The insoluble material was removed by centrifugation, and the supernatant was chromatographied on Express-Ion Exchanger C (2.5×30 cm) using step-wise elution with 0.01, 0.02, 0.05, 0.1 and 0.2 M NH_4HCO_3 at pH 8.0. The fractions at each NH_4HCO_3 concentrations were lyophilized, and then later found the fraction (45 mg) eluted at 0.02 M NH_4HCO_3 showing stimulatory effects on insulin secretion was further purified.

With those peptide samples, an aliquots 2.0 mg were subjected to the first reverse-phase HPLC on TSK ODS 120-T (10 µm, 7.8×300 mm). Eluent A was 0.1% trifluoroacetic acid (TFA) in water, and B, 0.1%, TFA in acetonitrile and a linear gradient of 15 to 65% B in 50 min (l ml/min) was used. The fraction absorbing at 214 nm were collected and lyophilized. The fraction containing the peptides with stimulating activity was further purified by the second HPLC, and the results are shown in Figure 3. The compound corresponding peak 1 is identified as $Valosin_{3-25}$, peak 2 as GIP_{1-39} and peak3

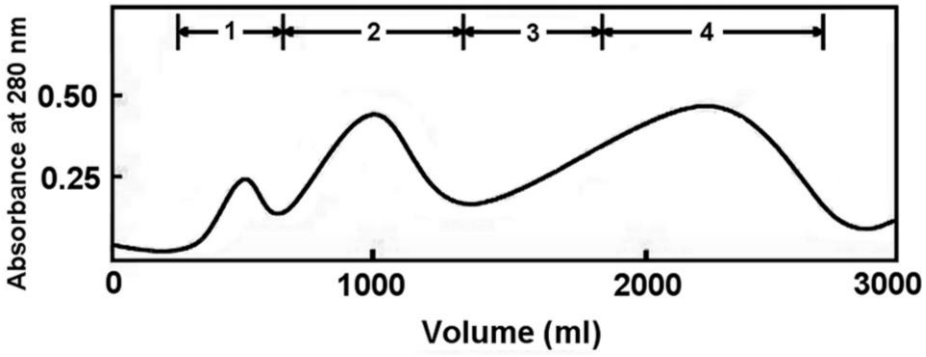

Figure 2. Results of purification. Exclusion chromatography of the crude peptides on Sephadex G-25 in 0.2 M acetic acid. Fraction 4 was collected and lyophilized (cf. text).

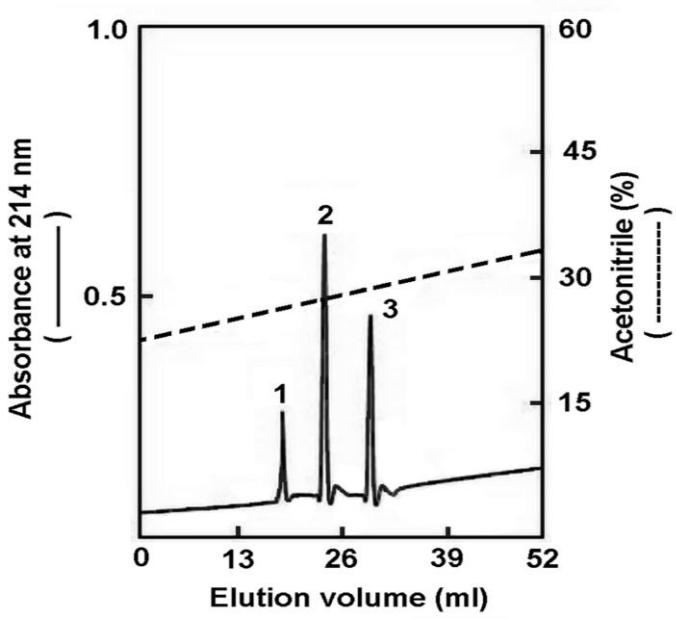

Figure 3. The final HPLC purification, peak 1. Valosin$_{3-25}$, 2. GIP$_{1-39}$, and 3. PEBP$_{93-124}$.

PEBP$_{93-124}$, respectively.

Peptide purification

In this study, the crude materials for peptide preparations were the concentrate of thermostable intestinal polypeptides (CTIP) which was produced as described in references (Xie et al., 2004; Chen et al., 1988). The water-miscible organic solvent isopropanol was employed at early stage for fractionation of peptides. The exclusion chromatography on Sephadex G-25-fine (Pharmacia, Uppsala, Sweden) column (10×90 cm) was used. Ion-exchange chromatography was carried out on Express-Ion Exchanger C (Whatman International Ltd, England) column (2.5×30 cm). Reverse-phase high-performance liquid chromatography (HPLC) utilized a Waters (Milford, Mass.) system with detection at 214 and 280 nm, fitted with TSK-ODS 120 T column (10 µm, 7.8×300 mm) and Vydac 218TP54 column (C 18, 4.6×250 mm). The peptide purity was analyzed by SDS-PAGE.

Amino acid composition analysis

The total amino acids compositions were determined with Pharmacia alpha plus 4151 ninhydrin-based analyzer after hydrolyzation of sample at 110°C for 24 h in evacuated tubes with 6 M HCl containing 0.5% (W/V) phenol.

Mass spectrometry determination

Molecular masses were determined using a Plasma-Desorption

Table 1. The amino acid sequences of the $PEBP_{93-124}$, and $Valosin_{3-25}$.

Peptide	Amino acid sequence	Residues	Determined Mw	Theoretial Mw
$PEBP_{93-124}$	KGNDISSGTVLSDYVGSGPPKGTGLHRYVWLV	32	3359.8	3360.77
$Valosin_{3-25}$	YPVEHPDKFLKFGMTPSKGVLFY	23	2699.5	2701.18

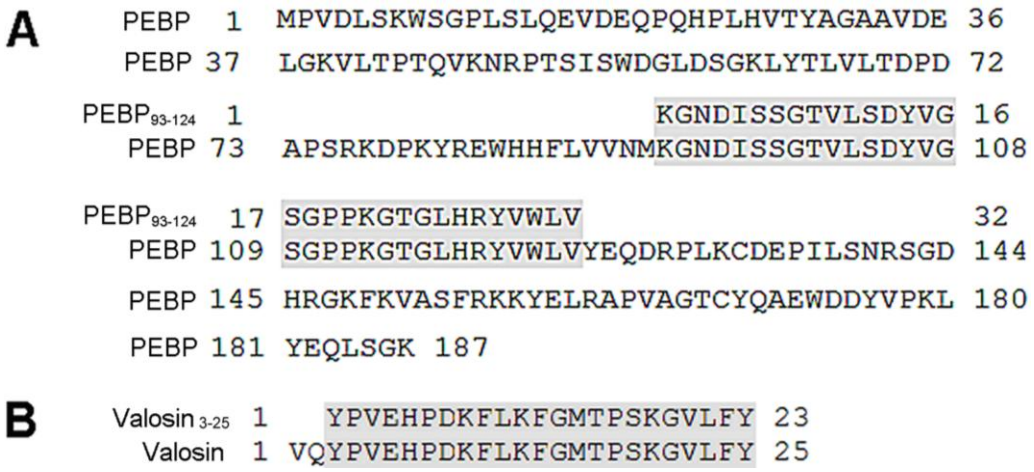

Figure 4. Alignment of $PEBP_{93-124}$ and $Valosin_{3-25}$ with their parental protein from porcine.

Time-of-Flight Mass Spectrometer (Biolon 20). Samples were dissolved in 0.1% trifluoroacetic acid containing 20% acetonitrile, applied to aluminium foil covered by nitrocellulose and dried in a stream of nitrogen. Data were accumulated (usually 10^6 counts) at 15 kV acceleration voltage. H^+ and Na^+, or nitrous oxide, were used as internal standards for calibration.

Amino acid sequence analysis

Edman degradation was used for peptide sequence analysis which were carried out with an Applied Biosystems 477A instrument coupled to a 120 A analyzer, a Milligen 6600 instrument with a Waters 440 analyzer, or an Applied Biosystems 470A instrument with separate HPLC phenylthiohydantoin determination.

The effects of peptides on insulin secretion

The effects of the isolated peptides on glucose-induced insulin secretion were tested in an isolated rat pancreatic islet perifusion system. The detail procedures referred our previous paper (Ostenson and Grill, 1986; Bowe et al., 2012). In brief, male Sprague–Dawley rats (200–250 g) were sacrificed in anaesthesia and the pancreas was removed and then gently digested with collagenase. The islets were cultured overnight at 37°C under 5% CO_2 in medium RPMI 1640 containing 11 mM glucose and 10% heat-inactivated calf serum. The insulin released was tested in granule of three islets as discribed in (Xie et al., 2004). The incubation medium contained 3.3 or 16.7 mM glucose respectively, with or without 10 nM peptide. Radioimmunoassay was used to determine the amount of released insulin in the medium (Xie et al., 2004a, b).

Statistical analysis

The data are presented as mean±SEM. Significance was tested by Student's *t*-test, and differences in the mean values were considered significant at a probability of $P<0.05$.

RESULTS

Molecular weight and amino acid sequence

The mass weight of the $PEBP_{93-124}$ and $Valosin_{3-25}$ were determined by mass spectrometry and the amino acid sequences were determined by Edman degradations which were shown in Table 1. The alignment of the fragments with their parental protein from porcine was shown in Figure 4.

The function of peptides on insulin secretion

The three peptides purified as described above were dissovled to 10 nM in 0.01 M PBS, and then tested the effect on insulin release from isolated pancreatic β cells in the presence of 16.7 mM glucose. The standard sample of porcine GIP_{1-42} (gift from the Department of Medical Biochemistry and Biophysics, Karolinska Institute of Sweden, originally lyophilized and kept at -80°C) was comparably ascertained on insulin secretion (n=15). As

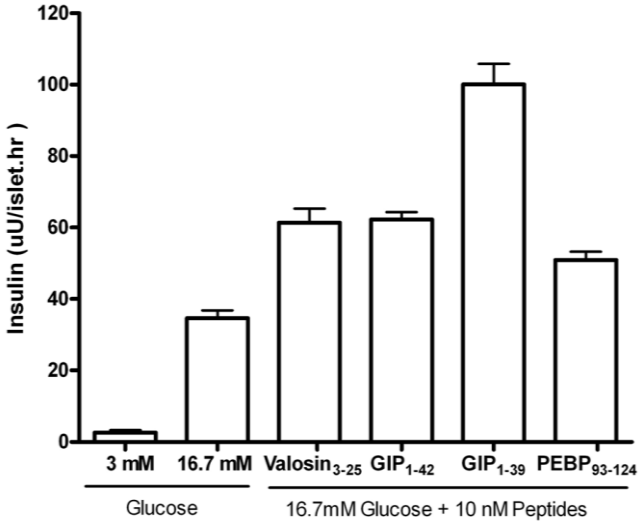

Figure 5. Comparison the effects of the peptides on insulin secretion. Data are represented as means ± S.E.M. from 15 experiments (P<0.001).

reported (Xie et al., 2004), the GIP_{1-39} showed higher activity than GIP_{1-42} (*P<0.01*). The novel fragments, $PEBP_{93-124}$ and $Valosin_{3-25}$, both stimulated insulin secretion from β cells which were similar to the GIP_{1-42}, and much higher activity than 16.7 mM glucose (*P<0.01*) (Figure 5).

DISCUSSION

Many gastrointestinal proteins / polypeptides demonstrating enterogastrone activity are important and continuously detected. In this study, we purified and chemically characterized the truncated fragments of three gastrointestinal polypeptides. The first one is a 32-residue $PEBP_{93-124}$. The intact PEBP is a 187 amino acids protein which was deduced from the human and bovine cDNA, and showed that both of the species share 96% amino acid identity (Perry et al., 1994). The PEBP also was identified as hippocampal cholinergic neurostimulating peptide (HCNP) and was cloned from rat brain cDNA (Tohdoh et al., 1995). The $PEBP_{93-124}$ is cleaved after the N-terminal 92nd residue methionine of the intact PEBP (HCNP), and the 20 to 32-residue fragment of $PEBP_{93-124}$ covered the partial consensus sequence, R/K-X_{2-3}-G-X_3-(hydrophobic)$_4$-X_2-D/E comprised residues 112 to 125 of the rat and human HCNP precursor proteins (Perry et al., 1994; Tohdoh et al., 1995). In this study, $PEBP_{93-124}$ had insulinotropic activity (Figure 5) that was totally different from its parental protein PEBP, which demonstrates that HCNP precursor proteins could be processed to different functional peptides.

The second one is a 23-residue $Valosin_{3-25}$. Valosin was isolated from porcine intestine and structurally determined as 25 amino acid residues, and has effects on the digestive system of dogs (Schmidt et al., 1985). Valosin Containing Protein (VCP) was late found by a cDNA approach and smaller than the predicted product of the cDNA sequence (Koller and Brownstein, 1987). Interestingly, the gene of VCP was also later cloned from the human hypothalamus-pituitary-adrenal axis (Hu et al., 2000) which shows Valosin and its possible processed peptides might be function forms in nervous system. We purified the $Valosin_{3-25}$ with N-terminal Tyrosine, just truncated the valine (V) and glutamine (Q) from the parental Valosin (Figure 4B). Our results showed that $Valosin_{3-25}$ had significant insulinotropic activity similar to the GIP_{1-42} (Figure 5) which showed the $Valosin_{3-25}$ might contribute to the digestion system of porcine.

The bioactivities that the two truncated peptides showed were not reported in their parental proteins yet, which indicated that the post-translational process for proteins was very important for its functions. These results proved that to isolate and characterize proteins from the natural materials in the post genomic and bio-informational era are still necessary, which will help us to have a deep insight into proteomics. This work may contribute to the further understanding of the relationships between proteome and genome in the post genomic and bio-informational era. In addition, these purified may provide an option to treat Diabetes and bring a light to understanding the mechanism of Diabetes.

Conclusion

The two novel truncated gastrointestinal polypeptides were purified. The first one is a 32-residue $PEBP_{93-124}$. $PEBP_{93-124}$ had insulinotropic activity that was totally different from its parental protein PEBP, which demonstrates that PEBP (HCNP) precursor proteins could be processed to different functional peptides. The second one is a 23-residue $Valosin_{3-25}$. We purified the $Valosin_{3-25}$ with N-terminal Tyrosine, just truncated the valine and glutamine from the parental Valosin. $Valosin_{3-25}$ had significant insulinotropic activity similar to the GIP_{1-42}. The both truncated peptides showed insulinotropic activity, that implied truncated peptides might play an important role in the balance of blood glucose.

ACKNOWLEDGEMENTS

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