ISOLATION OF LEPTIN-BINDING PEPTIDES FROM A RANDOM PEPTIDE PHAGE LIBRARY

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Abstract

Leptin plays a role in regulating the body weight in mice. Injection of recombinant mouse leptin expressed in *Escherichia coli* reduced the food intake and body weight in normal, ob/ob and diet-induced obesity mice. Hyperglycemia, hyperinsulinemia and hypothermia can also be corrected in ob/ob mice after leptin injection. Leptin is a 16 kDa secretory protein comprising of 167 amino acids produced in adipose tissue and is secreted to blood stream. In this study, a recombinant mouse leptin was generated and purified from a baculovirus expression system. This protein was used to identify putative ligands using a phage library of random peptides. Three leptin-binding phage clones were found which were characterized by DNA sequencing and ELISA methods. The amino acid sequences of the reactive peptides are: LAYCSDPVRCLVWWY, MFWISAVSFVDHALV and LVLVLSAFLCCGVG. All three clones bound to recombinant human and mouse leptins. These peptides may be useful tools to study leptin-receptor interaction, food intake and body weight regulation.

Key words: leptin, baculovirus, phage library, peptide

INTRODUCTION

The mouse obese gene was first discovered in C57BL/6J ob/ob mice by positional cloning (1). This gene encodes a 16 kDa polypeptide called leptin which is expressed specifically in adipose tissues. The expression of leptin was dependent on the nutritional status, the hormonal status and the environmental temperature. Fasting (30 hours) or semi-starvation (10 days) reduced the expression of ob mRNA in rats (2) while refeeding fasting rats could increase the expression of ob mRNA to a level comparable to the fed control within 4 hours. (3). Feeding increased expression of leptin, which subsequently decreased the appetite and increased energy expenditure. Administration of exogenous insulin increased the ob mRNA expression in rat (2). When rats were exposed to cold environment ($6^{o}C$) for a day, the expression of ob mRNA in brown adipose tissue (BAT) decreased (2).

Recombinant mouse leptin when given intra-peritoneally to mice was shown to decrease both body weight and food intake in normal mice, the C57BL/6J ob/ob mice and diet-induced obesity (DIO) mice (4, 5, 6). Recombinant human leptin expressed in bacteria also had similar effects (4, 7). However, no significant reduction in body weight and food intake were detected in db/db mice. The decrease in the food intake and body weight in the responsive mouse strains were dose-dependent (5, 6). Leptin also involved in reproductive function. Injection of recombinant leptin enhanced gonadotrophin levels, follicular development and fully restored fertility in ob/ob mice. Moreover, earlier sexual maturation, copulatory plug, vaginal opening, mating and estrus behaviours appeared after leptin injection (8, 9).

Leptin belongs to the family of helical cytokines (10). The result of threading analysis also indicated that the structure of leptin resembles the family of helical cytokines (11). A cDNA expression library was prepared from mouse choroid plexus

and a cDNA expressing leptin receptor (OB-R) was screened by a leptin-alkaline phosphatase (AP) fusion protein. The receptor is a 894 amino acid residues-long single membrane-spanning polypeptide initiated with a methionine and followed by a typical hydrophobic signal peptide for peptide secretion (12). Mouse OB-R was expressed in different tissues including choroid plexus, hypothalamus (12, 13, 14), lung, kidney (12), ovary, uterus, testis and anterior pituitary (14). Anatomical localization of OB-R in mouse brain showed that specific binding of (^{125}I) leptin was found in choroid plexus located in the dorsal part of the third ventricle and lateral ventricle (13) to which a number of other peptide hormones like insulin and vasopressin also bind (15, 16).

In the present studies, three leptin-binding peptide phages were selected from a 15-mer peptide phage library. In this phage library, random peptide sequences of 15 amino acid residues in length are displayed on the phages as Gene III product fusion proteins. The specific bindings of the three peptide phages to leptin were confirmed by dose-response ELISA assays and self-inhibition ELISA assays. The specific bindings of the selected phage peptides to the recombinant mouse and human leptins expressed in bacteria and the recombinant mouse leptin expressed in insect cell were also investigated. These phages may be used as ligands to purify leptin by affinity chromatography and to detect leptin in ELISA.

EXPERIMENTAL

Chemicals and reagents. Anti-M13 antibody conjugated with horseradish peroxidase (HRP) and AutoCycle™ Sequencing kit were purchased from Pharmacia. BaculoGold™ baculovirus expression system and *Sf*21 insect cell were obtained from Pharmingen. DE32 anion exchanger was purchased from Whatman. ELISA plates (ImmunoPlate MaxiSorp™) was the product of Nunc. *o*-phenylenediamine dihydrochloride (OPD) were purchased from Sigma (St. Louis, MO, U.S.A.). Polyethylene glycol (PEG) 8000 were purchased from Sigma. Recombinant mouse and human leptin expressed in bacteria were purchased from Research Diagnostics Inc. SF900II serum-free medium was from Life Technologies. Tosyl activated dynabead was bought from Dynal Inc. 3,3',5,5'-tetramethylbenzidine (TMB) substrate and stop solution were the products of Kirkegaard and Perry Laboratories.

Buffers. Phosphate buffered saline (PBS): 137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 and 8.1mM Na_2HPO_4, pH7.4. TBS buffer: 20mM Tris-HCl, 140mM NaCl, pH7.4. Buffers for peptide phage library screening -- Coupling buffer: 0.05M disodium tetraborate, 0.05M boric acid, pH 9.6. Wash buffer: 1xTBS, 0.5% (v/v) Tween-20, pH7.4. Glycine buffer: 0.1M glycine, pH11. Buffers for ELISA -- Coating buffer: 15mM Na_2CO_3, 35mM $NaHCO_3$, pH9.6. Wash buffer: 1xTBS, 0.05% (v/v) Tween-20, pH7.4. Blocking buffer: 1xTBS, 0.05% (v/v) Tween-20, 1% (w/v) BSA, pH7.4. PEG/NaCl solution: 16.7% (w/v) Polyethylene glycol 8000 and 3.3M NaCl. 0.05M Phosphate-citrate buffer for color development using OPD substrate: 51.4mM Na_2HPO_4, 24.3mM citric acid, 0.012%(v/v) H_2O_2 (added just before color development), pH5.0. Color stopping solution for OPD substrate: 25ul 3N HCl for 100ul reaction mixture.

Overexpression and purification of recombinant mouse leptin in insect cell. Mouse leptin cDNA was amplified from first strand cDNA obtained in adipose tissue of mouse using primers 5'-ATTAGAATTCATCCCAGGGAGGAAAATG-3' (sense) and 5'-AATAGAATTCTCAGCATTCAGGGCTAA-3' (antisense) in polymerase chain reaction (17). Then, recombinant mouse leptin was produced in *Sf*21 insect cell according to the instruction provided in BaculoGold™ baculovirus expression system (18). Recombinant leptin was expressed in SF900II serum-free medium and the clarified medium was dialyzed against 25mM Tris-HCl, pH 7.4 before loading into a DE32 anion exchange column. The recombinant leptin was then eluted with 25mM Tris-HCl, 40mM NaCl, pH7.4. The N-terminal protein sequence of the recombinant leptin was confirmed by the HPG1005A Protein Sequencing System (Hewlett-Packward).

Preparation of leptin-coupled dynabeads. Recombinant mouse leptin was first immobilized onto tosyl activated dynabeads and then the non-occupied sites were blocked by BSA. Briefly, 200ul tosyl activated dynabead ($4x10^8$ beads/ml) was washed 5 times with coupling buffer in a 1.5ml eppendorf tube. Then, it was resuspended in 200ul coupling buffer. 30ug purified recombinant mouse leptin was dissolved in 200ul coupling buffer and mixed with the dynabeads. The coupling was performed at 4^oC overnight on a roller. After coupling, the non-specific sites was blocked by BSA in blocking buffer. The leptin coupled dynabead was washed 3 times with 800ul 1% BSA in PBS buffer, pH7.4 for 10mins thrice. Then, it was resuspended in 200ul PBS buffer with 1% (w/v) BSA and stored at 4^oC overnight.

Selection of phages expressing leptin-binding peptides. For the selection, 200ul ($1.3x10^{10}$ pfu/ml) primary 15-mer peptide phage library (source: George Smith, University of Missouri-Columbia, MO, USA) in TBS buffer, pH7.4 was mixed with

50ul leptin coupled dynabeads at 4°C overnight. Afterwards, the beads were captured by a magnet and washed 10 times with 500ul wash buffer for 5 mins at room temperature. Then, the specific leptin-binding peptide phages were eluted with 500ul 0.1M glycine buffer, pH11 by rotating at room temperature for 30mins. Afterwards, the beads were captured by a magnet and the supernatant containing the specific leptin-binding peptide phage was dialyzed in two changes of 1L TBS buffer, pH7.4 at 4°C for 5 hours and then overnight (Primary selection). In another attempt, the phages eluted in the primary selection were mixed with the leptin coupled dynabeads again to generated the secondary selection.

Isolation of single phage particle. Individual phage particles were obtained by infecting *E. coli* strain K91 cell with the eluted phage. 2ul eluted phages were added to 50ul *E. coli* K91 overnight culture in a 1.5ml eppendorf tube. After incubated at 37°C for 1hr, 450ul LB medium was added to the tube and 100ul culture was spread onto five LB plates containing 20ug/ml tetracycline for selection. After overnight incubation, individual colonies on the plates were inoculated into 2ml LB broth with 20ug/ml tetracycline in 15ml falcon tubes. The cultures were shaken at 37°C overnight at 250rpm/min. The bacterial cells were removed by spinning at 3000rpm at 4°C for 20mins and the phage-containing supernatant was collected in 2ml screw-capped tube. To each ml of overnight culture supernatant, 150ul PEG/NaCl solution was added and the phages were concentrated by spinning at 14,000rpm at 4°C for 15mins after overnight incubation. The concentration of the phage particles was determined by reading optical density, where the no. of phage/ml = (A269nm-A320nm)x(2x 10^{14})/30.

Enzyme linked immunosorbent assay (ELISA). The phages displaying leptin-binding peptides were screened by ELISA (19). 100ul recombinant leptin (3-5ug/ml in coating

buffer), BSA (3-5ug/ml in coating buffer) or coating buffer were coated onto the wells of 96-well Maxisorp™ ELISA plate at $4^{o}C$ overnight. After washing, the non-specific sites were blocked by 200ul/well blocking buffer overnight. After washing the wells thrice with wash buffer, $2.5x10^{10}$ phage particles in 100 ul wash buffer were added to the well. After incubating at $37^{o}C$ for 1hr and washing, 100ul (diluted 1:5000) anti-M13 antibody conjugated with horseradish peroxidase, HRP was added to each well and incubated at $37^{o}C$ for 1 hr. Finally, 100ul TMB or OPD substrate was added at room temperature. The reaction was stopped by adding 100ul TMB stop solution or 25ul 3N HCl and the absorbance was measured at wavelength 450nm or 490nm.

Four ELISA experimental setups were performed to characterize the binding between peptide phages and leptin. The four experimental setups included (1) a titration of phage concentrations and ELISA signals, (2) the dose response effect of leptin coating, (3) a self-inhibition study using excess BSA and leptin as inhibitors and (4) binding assays of the peptide phages to human and mouse leptins. In the first experimental setup, increasing phage number ranging from $1x10^{8}$ to $2.5x10^{10}$ phage was added to each well after discarding the blocking solution. In the dose-response study, 100ul 0.01, 0.1, 0.5, 1 and 3ug/ml leptin or BSA were coated onto the wells. In the self-inhibition study, 0, 1, 5, 20, 30 and 50ug/ml inhibitory protein (either leptin or BSA) were mixed with $2.5x10^{10}$ phage particles in the wells coated with leptin (3ug/ml). And in the last experimental setup, recombinant human and mouse leptins produced in bacteria and recombinant leptin produced in insect cell were coated on the ELISA plate. All these experiments were repeated thrice. All samples were done in duplicate or triplicate wells. Standard derivations were calculated and incorporated in the figures in the result section.

DNA sequencing. DNA sequencing procedures (20) were carried out following the instruction of AutoCycle™ Sequencing kit (Pharmacia). The primer, fuse-S35, 5'-CCC TCA TAG TTA GCG TAA CG-3' and radioactive dCTP were used. To prepare the sequencing template, phage particles precipitated by PEG/NaCl solution from 1 ml overnight culture was resuspended in 200ul TBS buffer, pH7.4 and extracted with 200ul phenol, pH 8.0. After vortexing for 30s and centrifugation, the extracted DNA in the aqueous layer was precipitated by absolute ethanol. Finally, the pellet containing 2.5ug DNA was resuspended in 8ul autoclaved water for DNA sequencing.

RESULTS

Overexpression and purification of recombinant mouse leptin in insect cell. The full length mouse leptin cDNA was successfully amplifed from the adipose tissue and a recombinant baculovirus expressing leptin was generated from it using a baculovirus expression system. Large amount of recombinant leptin was produced by *Sf*21 cell in serum-free medium, SF900II, by infecting suspension culture at MOI 0.04. The recombinant mouse leptin (16 kDa) was secreted extracellularly and its signal peptide was shown to be cleaved after secretion by N-terminal protein sequencing. Highly purified recombinant leptin was obtained by DE32 anion exchange chromatography (Figure 1). Then, the purified recombinant leptin was used for the selection of leptin-binding peptide phages. The first twenty N-terminal amino acid residues were sequenced which confirmed the cleavage of the signal peptide at the predicted site (1).

Isolation and characterization of leptin-binding peptide phages. Sixty individual phages from primary selection and 14 individual phages from secondary selection were screened by ELISA. Only three clones giving the highest signals, 1-56, 2-3 and 2-7, were shown to have specific binding to leptin but not to BSA nor the plate. An ELISA was performed to study the minimal number of phages required to generate ELISA signals (Figure 2). Different phage number ranging from 0 to $2.5x10^{10}$ phage particles were added to the well and the signals of phages 1-56, 2-3 and 2-7 were increasing with the phage number. No such tendency was noted for the negative control phage 1-1. Thus, $2.5x10^{10}$ phage number were generally used in the subsequent tests. Fixed amount of phages, 1-56, 2-3 and 2-7, were tested in a dose-response study to determine if their binding to leptin was specific. The results showed that as the amount of coating leptin increased, the binding of leptin-binding peptide phages to the plate also increased. However, increasing the amount of BSA coating on

ELISA plate gave no increase in binding which suggested that the binding to leptin was specific (Figure 3). Afterwards, a self-inhibition test was performed to study the binding of phages 1-56, 2-3 and 2-7 to leptin (Figure 4). All three phages showed inhibition when incubated with 5ug/ml free leptin. Moreover, when the phages were incubated with 50ug/ml free leptin, only 50% signal were obtained for 2-3 and 2-7 and less than 40% signal was obtained for 1-56. These results provide evidence for specific binding of 1-56, 2-3 and 2-7 to leptin.

The binding of phage 1-56, 2-3 and 2-7 to different kinds of recombinant leptins including recombinant mouse leptin produced by insect cell, recombinant mouse leptin and human leptin produced in *E. coli*, were studied (Figure 5). The results showed that all three phages could bind to all three types of recombinant leptins but not to the negative control BSA. For phages 1-56 and 2-3, slightly stronger signals were found in mouse leptin produced by insect cell than that produced in *E. coli*. However, for phage 2-7, significant (Student t-test, $p<0.005$, two tails, unpaired) stronger signal was generated from mouse leptin produced in *E. coli*. Theoretically, these two recombinant proteins should be functionally and structurally identical. However, in practical, the recombinant mouse leptin produced in insect cell was a soluble protein, whereas mouse leptin produced in *E. coli* was in the form of inclusion bodies, which required solubilization, denaturation and refolding to restore its activity. This difference may affect the coating efficiency of the protein onto the ELISA plate and the binding affinities of various phage peptides. Nonetheless, the experimental results showed that all three peptide phages were capable of binding to both recombinant mouse and human leptins.

Finally, the sequences of the peptide displayed on the leptin-binding phages were determined by DNA sequencing. The cDNA sequences and the deduced amino

acid sequences of 1-56, 2-3 and 2-7 were shown in Figure 6. It was found that 1-56 and 2-3 displayed peptides with 15 amino acid residues whereas 2-7 displayed a peptide with 14 amino acid residues.

DISCUSSION

In order to select leptin binding peptide phages, recombinant mouse leptin expressed in baculovirus was used. Since recombinant mouse leptin produced in insect cell was soluble, it should be a better ligand than the recombinant mouse leptin produced in *E. coli.* In *E. coli*, recombinant leptin was produced as an insoluble protein in the inclusion bodies, which required denaturation and refolding to recover its activity. During the denaturation process, the polypeptide was solubilized by chaotropic agents such as 8M Urea. 100% refolding is not attainable and misfolded protein may expose certain hydrophobic regions that might cause non-specific binding (21). Therefore, the soluble recombinant mouse leptin produced in insect cell was used for the selection of leptin binding peptide phages in this study.

To determine the minimal concentration of phage particles required for the generation of ELISA signal, a titration of phage concentration was carried out and the results were presented in figure 2. From 0 to $1x10^{10}$ phage, the increase in the signal was not profound. On the other hand, when the number of phages was increased from $1x10^{10}$ to $2.5x10^{10}$, O.D.$_{450nm}$ increased over 0.5. Since each well contained 0.1 ml volume, the concentration of the phage particles were $2.5x10^{14}$/litre, which was equivalent to 0.415 nM. In order to show that the ELISA signals were not due to non-specific binding of the phages to the ELISA plate, the dependance of ELISA signals on increasing coating of leptin or BSA was examined in a dose-response study. Figure 3 showed that the ELISA signals increased with increasing amount of leptin coated but not with BSA coated on ELISA plates. This indicated that the selected phage clone 1-56, 2-3 and 2-7 were specifically bound to leptin coated on the plate but not to the plate itself. This was further confirmed by the self-inhibition studies. The result of self-inhibition studies showed that free leptin can inhibit peptide phage binding to

the coated leptin. 20ug/ml free leptin gave more than 20% inhibition in all three peptide phage clones. When free leptin was increased to 50ug/ml, 64.6%, 48.0% and 49.8% inhibition were obtained for clones 1-56, 2-3 and 2-7 respectively (Figure 4). At the same concentratons, BSA did not inhibit the binding of the three peptide phage clones to the coated leptin.

As the three phage peptides were selected by their abilities to bind mouse leptin, their bindings to human leptin were also examined. The results shown in figure 5 indicated that all three peptides were able to bind to human leptin but not to BSA. This is not unexpected as there is 84% homology in amino acid sequences between human and mouse leptins. In this study, the leptin cDNA was obtained from mouse adipose tissue because it was more accessible than human adipose tissue. As the homology between human and mouse leptin was very high, it was expected that the majority of peptide phages selected from mouse leptin could cross-reacted with the human leptin. This was shown to be the case in this study.

The leptin-binding peptide phages identified in this study have several potential applications. The specific binding of the peptide phages to leptin can be used to purify recombinant leptin in affinity chromatography. In a previous report (21), recombinant leptin generated from *E. coli* was purified by anion chromatography or by combination of ion-exchange, hydrophobic interaction chromatography and gel filtration (22). Thoerectically, leptin can also be purified by affinity chromatography using these selected leptin-binding peptide phages which can be produced in large quantity by overnight culture. They can also be used for the detection of leptin in ELISA and for the study of the binding site of leptin to its receptor. If the peptide phages interfere with leptin binding to its receptor, a state of leptin deficiency might

be induced if the phages are injected into animals. These phages may be useful tools to study leptin, food intake and body weight regulation.

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FIGURE LEGENDS

Figrure 1. Recombinant mouse leptin produced by *Sf*21 insect cell and purified by DE32 anion exchange chromatography. Recombinant mouse leptin was bound to the column whereas unbounded proteins were washed away by running buffer. The bound recombinant mouse leptin was eluted by elution buffer, (25mM Tris, 40mM NaCl, pH 7.5). Protein samples (20ul elutant) were run into a 12% SDS-PAGE gel and stained by Coomassie Blue. Lane 1: Purified recombinant mouse leptin without visible impurities, lane 2: Protein marker.

Figure 2. Increasing signals were generated by increasing the number of phages in selected phage clones 1-56, 2-3 and 2-7, but not in the negative phage clone 1-1. ELISA plates were coated with 0.3ug / well leptin and blocked with 1% BSA at 4°C. Then, different number of phage ranging from 0 to $2.5x10^{10}$ phage particles were added to the wells and incubated at 37°C for 1 hour. Anti-M13 conjugated with horseradish peroxidase was added to the wells and TMB was used to develop the color for 10min. Relationship between the number of phage and O.D. $_{450nm}$ of the selected peptide phages were shown in the figure.

Figure 3. Dose-response curve was produced by plotting O.D.$_{490nm}$ against coating protein concentration. $2.5x10^{10}$ phage particles were added to the well coated with increasing amounts of leptin or BSA. More leptin coating increased the signals of 1-56, 2-3 and 2-7 but not of the negative phage clone 1-20. However, the increase were not found in the wells coated with BSA.

Figure 4. Relationship between the % binding signal and the concentrations of inhibitory protein. Six different concentrations (0, 1, 5, 20, 30 and 50ug/ml) of inhibitory proteins (either leptin or BSA) were used. Even at the highest concentration (50ug/ml), BSA gave no inhibition. Higher degree of inhibition were found in all three peptide phages when the concentration free leptin increased. At 50ug/ml free leptin, only 50% signals were obtained for clones 2-3 and 2-7 and only 40% signal was obtained for clone 1-56.

Figure 5. The binding of peptide phages to various kinds of recombinant leptin. 100ul 3ug/ml of recombinant mouse leptin produced in insect cell and recombinant mouse and human leptin produced in *E. coli* were coated onto the wells of ELISA plates. Then, the non-specific sites were blocked by BSA before $2.5x10^{10}$ phages were incubated in the plates for 1hr at $37^{o}C$. Unbounded phages were washed away and anti-M13-HRP was added. Finally, substrate was added to the wells and absorbance was recorded. The readings were the mean value of triplicates.

Figure 6. cDNA and amino acid sequences of peptide displayed on phages.

Figure 1. Purification of recombinant mouse leptin by dimethyl aminoethyl (DE) anion exchange chromatography

Figure 2. The ELISA signals produced by using different number of phage

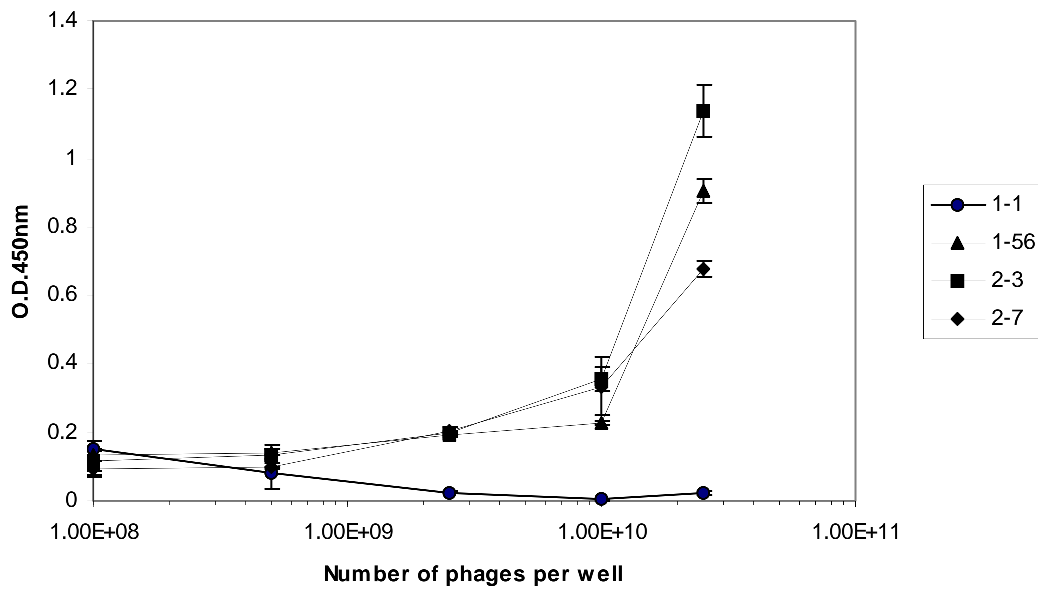

Figure 3. Dose-response effect of leptin coating on the binding of leptin-binding phages

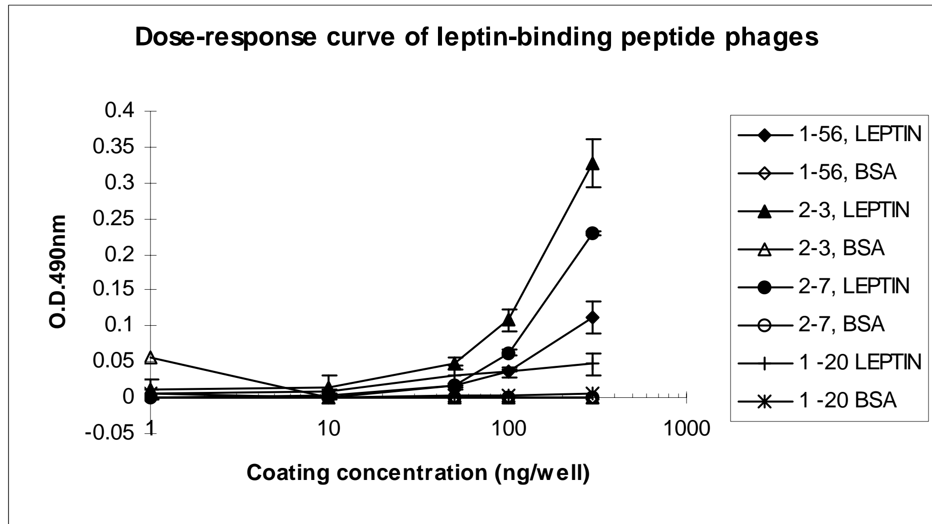

Figure 4. Self-inhibtion test on 1-56, 2-3 and 2-7

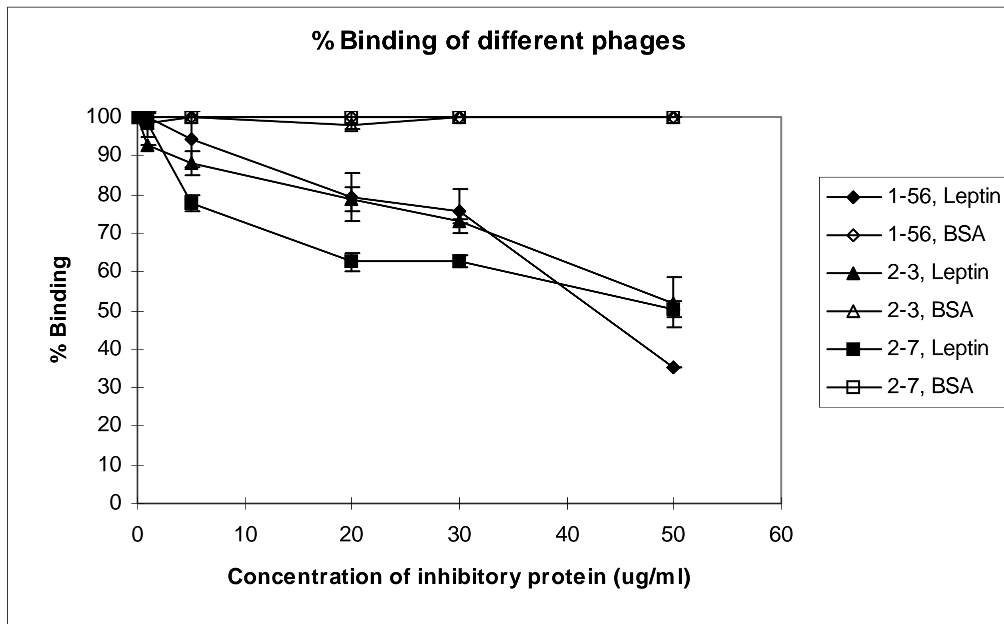

Figure 5. Binding of the peptide phages to various kinds of recombinant leptins

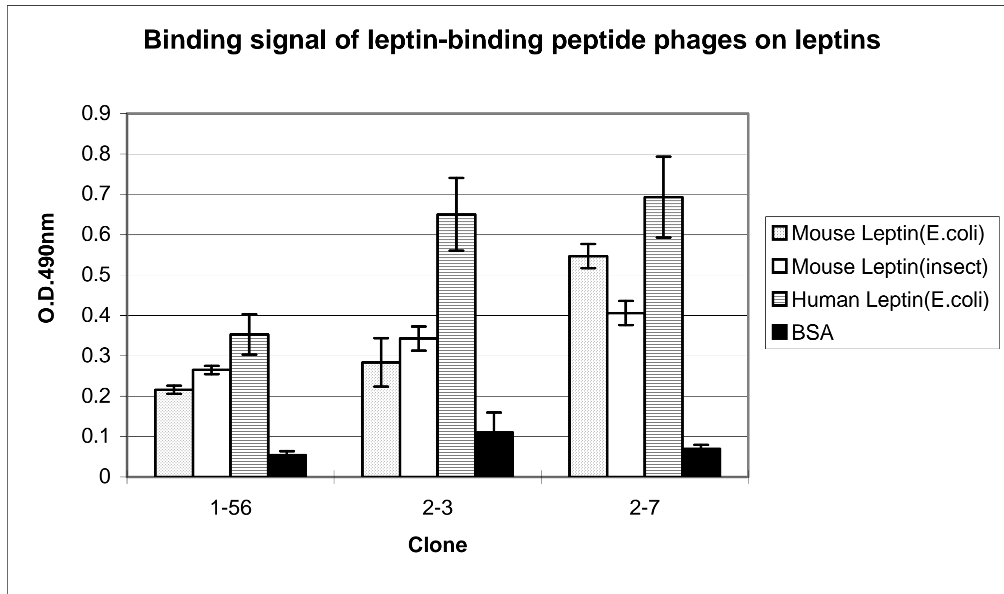

Figure 6. cDNA and translated amino acid sequences of peptide displayed on phages

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1-56     CTT GCT TAT TGT AGT GAT CCT GTG CGG TGT
          L   A   Y   C   S   D   P   V   R   C
         CTT GTT TGG TGG TAT
          L   V   W   W   Y

2-3      ATG TTT TGG ATT TCT GCT GTG TCG TTT GTT
          M   F   W   I   S   A   V   S   F   V
         GAT CAT GCT CTT GTG
          D   H   A   L   V

2-7      TTA GTG CTA GTT TTG TCG GCG TTT CTT TGT
          L   V   L   V   L   S   A   F   L   C
         TGT GGT GTG GGT
          C   G   V   G
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ABBREVIATIONS USED

BSA, bovine serum albumin; DE, dimethy aminoethyl; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LB, Luria-Bertani broth; PBS, phosphate buffer saline; PEG, polyethylene glycol; TBS, tris buffer saline; TMB, 3,3’,5,5’-tetramethylbenzidine and Tw-20, Tween-20.

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