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Selection of Trypsin Inhibitors in Phage Peptide Library[1]

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The newly developed techniques of peptide libraries have become a conventional and efficient method in screening ligands of proteins of interest. We present here the successful results of selection of trypsin inhibitors in a phage hexapeptide library. After affinity selection and activity assay, peptide sequences, deduced from DNA sequencing of the phage peptides with the most striking trypsin activity, share some common features with trypsin inhibitors reported. All of the phage peptides selected out and those native and synthetic trypsin inhibitors reported are composed of three parts: (a) positively charged part (Arg, Lys or their analogs); (b) polar part that may form hydrogen bonds with Ser in the active site of trypsin; (c) hydrophobic part that interacts with the nonpolar region of trypsin active site.

Regulators of enzymes and cellular receptors have always been attractive candidates of therapeutic agents. Scientists even more concentrate on searching for small molecules as possible medicines by tailoring natural regulators or by de novo design according to enzyme or receptor structures already known. But these works are always tedious and limited on the study of structure-function relationship. The newly developed techniques of peptide libraries had been proved powerful tools in many cases in selection of small peptide ligands to native ligates including enzymes (1,2), antibodies (3,4,5,6), and some cellular receptors (7,8,9). As the technique depends on random screening of billions of chemical structures with no known structural similarity to the native ligands, it is essential to design sensitive screening method for successful selection.

In this paper, we designed a model basing on selection of trypsin inhibitors in a phage peptide library to prove the probability and efficiency of selecting therapeutic agents in peptide library. First, we collected trypsin binding recombinant phages according to the method described by Scott & Smith (10). Second, we use recombinant phages directly to analyze the effect of phage bearing peptides on trypsin activity. Then, we obtained tens of trypsin inhibitors successfully. The use of phage peptide directly in functional assay greatly reduce the time and money spent on peptide synthesis that had been considered unavoidable for functional assay before. But it may need further investigation on other enzymes and receptors to determine whether it can totally replace free peptides in other functional assay methods. To our knowledge, only the work of using phage peptides as antigen to immune animals directly has been reported (11,12).

MATERIALS AND METHOD

a. Affinity-selection in a phage hexapeptide library. Phage hexapeptide library generously rewarded by Scott Smith is a collection of hexapeptide displayed near the N-terminal of fd phage minor coat protein III. The peptide sequence of the N-terminal part of pIII is:

NH_2-Ala-Asp-Gly-Ala-*X6*-Gly-Ala-Gly-

(X6 indicate the degenerate peptides inserted). Phages with high affinity to trypsin were selected out after two rounds of biopanning by the method described by Scott & Smith (10).

The first round was performed by the method P+LS: Firstly, the ligand protein trypsin was biotinylated, while strepavidin was absorbed to a petri dish and cultivated over night at 4°C. Then trypsin was immobilized on the petri dish by binding

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to strepavidin. Thirdly, peptide library with titre around 10^{12} TU/ml was added and let to stay overnight at 4°C. Unbound phages were discarded by washing with TBS/Tween (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.5% v/v Tween 20). In the last step, phages bound to trypsin was eluted with elution buffer (0.1N HCl-Glycine pH 2.2, 1mg/ml BSA).

The second round of biopanning was performed by the method PL+S: Phages collected in the first round were propergated by infect K91, a male strain of E. coli, to give a titre around 10^{11} TU/ml in 200 ul TE, followed by incubated with 200 ul 0.08 mg/ml biotinylated trypsin over night at 4°C. Then, this mixture was moved to a strepavidin coated petri dish to bind with biotinylated trypsin absorbed. Then, phages bound were eluted.

b. Trypsin inhibition assay. The trypsin inhibitory activities of selected phages were analysized basing on the method described by Schwert (13).

Trypsin activity assay was carried out at 25°C. 200 μl trypsin (0.004 mg/ml dissolved in 0.001N HCl) and 50 μl selected phages qurifed from one clony was added into a 1 cm sample cell. After incubated at 25°C for 30 min, 2.8 ml N-benzoyl-L-arginine ethyl ester (BAEE) (30.9 mg BAEE, 280 mg $CaCl_2 \cdot 2H_2O$, 566 mg Tris dissolved in 500 ml H_2O, pH 7.6) was added. The adsorbances were read at 253 nm every minute for five minutes. Wild type phage fl was used as control.

The inhibitory activity of phage peptide was defined as the change ratio of trypsin activity after incubated with recombinant phages. IC50 was defined as the phage titer when inhibit trypsin activity to 50%. Activity and IC50 were calculated using the formulation below. The results were the average dates of three parallel experiments.

$$\text{inhibitory activity}(\%) = \text{Activity phage/Activity fl} \times 100\%$$
$$\text{IC50(TU/ml)} = \text{phage titer} \times 50 / \text{activity}$$

c. Dissociation constant assay. The affinity of phages to trypsin was compared by dissociation constant (Kdiss) and the Kdiss was defined as follows:

$$[E{-}P] = [E] + [P]$$
$$K_{diss} = [E][P]/[E{-}P] = ([E]0{-}[E{-}P])\,[P] / [E{-}P]$$

Add 100 ul trypsin solution (0.4 μg/μ1 in H_2O) into each well of a 96-well assay plate, allow to stay overnight at 4°C, and collected trypsin solution in each well, washed three times with TBS/Tween, and blocked the wells with blocking solution (0.1M $NaHCO_3$, 5 mg/ml dialyzed BSA, 0.02% NaN_3), for 2 hr at 4°C. Discarded the blocking solution, washed the wells with TBS/Tween as described previously, then added 100 μl phage about 10^{10} TU/ml, allow to react overnight at 4°C. The following day, collected solution in each well, washed the wells with TBS/Tween, added 200 ul elution buffer to each well, rocked gently for 10 min, then moved 100 ul into a Ep tube containing 20 ul 1M Tris (pH 9.0) to adjust pH into the range 7∼8.5. The amount of trypsin absorbed onto each well was determined according to the changes of protein concentration after incubated (protein concentration was determined by Coomassie blue dye binding assay, Sedmak et al. 1997). And the concentration of E-P (Enzyme-phage complex) and P (free phages) was calculated basing on the phage titer in the solution after incubated over night and in the elution buffer respectively.

d. DNA sequencing. Eight colonies of active recombinant phages as trypsin inhibitors were selected out randomly. DNAs encode the hexapeptide inserted were sequenced with T^7 sequence Kit from Pharmacia to reveal the peptide sequences of phage peptide inhibitors.

RESULTS AND DISCUSSION

There is no significant improvement of recovery of the second round of selection comparing with the first round (table 1). We are not quite sure whether the tightest-bound phages had been significantly condensed. So we completed functional assay before DNA sequencing to elicit inactive ones.

We tested the activity of nearly 100 colonies of the last round of selection. About 2 thirds of them showed no obvious inhibitory activity to trypsin just as wild type phage fl. It proves that it is

TABLE 1
Recovery of Phages in Two Rounds of Biopanning

	First round of biopanning	Second round of biopanning
Titer of impute phages (TU)[a]	1×10^{11}	1×10^{11}
Titer of output phages (TU)[a]	3×10^{5}	5×10^{5}
Recovery (%)	3×10^{-4}	5×10^{-4}

[a] Titer of phages is defined as tetracycline-resistant transducing units.

TABLE 2
Trypsin Inhibitory Activity of Selected Clones

No.	Activity (%)	Titer (TU/ml)	IC50
1	60	1×10^{10}	8×10^{9}
2	60	2×10^{9}	26×10^{9}
3	40	5×10^{9}	6×10^{9}
4	40	1×10^{12}	1×10^{12}
5	40	5×10^{11}	6×10^{11}
6	40	6×10^{9}	8×10^{9}
7	40	8×10^{9}	1×10^{10}
8	20	1×10^{10}	3×10^{10}

necessary to select further by functional assay after biopanning. The activity of eight active colonies was listed in table 2. DNA sequencing reveals that two of them are the same as shown in table 3.

IC50 was defined as the titer of phages which inhibit trypsin activity to 50% other than mole concentration. It is because there are average five copy of fused pIII per phage particle and the mole concentration of phage peptides displayed on the N-terminal of pIII is hard to calculate basing on phage titer. But it is still safe to say that Ki of these inhibitory phage peptides are in the range of $10^{-12} \sim 10^{-9}$ M because the mole concentration of phage particles is around 10^{-13} M and has shown significant inhibitory activity.

Peptide sequences do not show any obvious consensus sequences but there is still some regularities. First, they are rich in highly hydrophobic amino acid residues (Ile, leu, Phe, Val, Pro, et al). Second, most of them contain Arg or Lys that may interact with Asp in substrat binding site of trypsin. These phage peptides could have been the substrate of trypsin and their inhibitory activities could have been because that they are competitive substrates of trypsin. But since the activity assay was carried out after the phages had been incubated with trypsin for 30 min, substrate of trypsin must have been thoroughly digested, and even if any was left the inhibitory activity could not be so great, so that these phage peptides must be inhibitors of trypsin. Third, nearly all of them have at least one polar amino acid residues besides Arg and Lys (Ser, Asn, et al.) that may form hydrogen bond with Ser. These characters are shared by most synthesized (eg. 3-alkosy-4-chloro-7-guanidinosisocoumarins 7, 9, 11, synthesized by Kam (14), and trypsin inhibitors selected in SPCL by Eichler (15)) and native trypsin inhibitors reported. Peptide sequences near the reactive site of trypsin inhibitors were list in table 4, the reactive site was underlined. As shown in table 4, those trypsin inhibitors reported are also of great variance as those selected in the phage peptide library. The reason may be that the active site of trypsin is more flexible than enzymes other than protease and the structures of its inhibitors are also more variable. Compared with the sequences

TABLE 3
Activities, Peptide Sequences and Frequencies of Phage Peptides

Peptide sequence	IC50	Frequency
V-R-S-M-L-A	8×10^{9}	1
K-I-C-L-A-N	2×10^{9}	1
R-I-M-G-V-S	7×10^{9}[a]	2
R-I-I-G-C-S	1×10^{10}	1
N-R-N-L-G-Y	1×10^{12}	1
P-V-M-L-I-S	6×10^{11}	1
N-A-L-F-V-R	3×10^{10}	1

[a] The average activity of clone Nos. 1 and 6.

TABLE 4
Peptide Sequence of Some Native and Synthetic Trypsin Inhibitors

	Peptide sequence
Bovine pancreatic inhibition	CKARII
Kazal inhibitor from bovine pancreas	PRIYNP
Trypsin inhibitor from corn	IPGRLP
Bowman-Birk inhibitor	TKSNPP
Trypsin inhibitors selected in SPCL(15)	Ac-AKIYRP-NH_2
	Ac-LTKIFT-NH_2

of these inhibitors, more Arg have been selected out because there are more codons encoding Arg than Lys.

CONCLUSION

As shown above, we have successfully completed the selection of trypsin inhibitors in phage peptide library. In this work, we use phage peptide directly in functional assay that have never been reported to our knowledge. Although, the figures obtained can't vividly reflect Ki of phage peptide inhibitors, but they does reflect inhibitory efficiency in some degree that is sufficient enough to elicit inactive colonies of phages, thus greatly reduces the work of DNA sequencing, peptide synthesis and other further research works to get precious results. Comparing peptide sequences obtained with trypsin inhibitors reported, we can find some feature shared by most of them: they are composed of three parts: a. positively charged part(Arg, Lys or their analogs); b. polar part that may form hydrogen bonds with Ser in the active site of trypsin; c. hydrophobic part that interact with the non-polar region of trypsin active site. These facts further proved that this work is successful.

Thus, we are confident to say that properly designed, phage peptide used directly in functional assay can be hopeful method in research works of phage peptide libraries.

REFERENCES

1. Owens, R. A., Gesellchen, P. D., Houchins, B. J., and DiMarchi, R. D. (1991) *Biochem. Biophys. Res. Commun.* **181**(1), 402–408.
2. Eichler, J., and Houghten, R. A. (1993) *Biochemistry* **32,** 11035–11041.
3. Felici, F., Castagnoli, L., Musacchio, A., and Jappelli, R. (1991) *J. Mol. Biol.* **222,** 301–310.
4. Arsenault, H., and Weber, J. M. (1993) *FEMS Microbiology Letters* **114,** 37–.
5. Michael, C. N. et al. (1993) *PNAS, USA* **99,** 10700–.
6. Boettger, V., and Lane, E. B. (1994) *J. Mol. Bio.* 1994; **235**(1), 61–.
7. Doorbar, J., and Winter, G. (1994) *J. Mol. Biol.* **244,** 361–369.
8. Lam, K. S., Lebl, M., Krchnak, V., et al. (1993) *Gene.* **137,** 13–16.
9. Goodson, R. J., Doyle, M. V., Kaufman, S. E., and Rosenberg, S. (1994) *Proc. Natl. Acad. Sci. USA.* **91,** 7129–7133.
10. Scott, J. K., and Smith, G. P. (1990) *Science* **249,** 386–390.
11. Willis, A. E., Perham, R. N., and Waith, D. (1993) *Gene* **128,** 19–83.
12. Minenkova, O. O. et al. (1993) *Gene* **128,** 85–88.
13. Schwert, G. W., and Takenaka, Y. (1955) *Biochem. Biophys. Acta* **16,** 570.
14. Kam, C-H., Fujikawa, K., and Powers, J. C. (1988) *Biochem.* **27,** 2547–2557.
15. Eichler, J., and Houghten, R. A. (1993) *Biochemistry* **32,** 11035–11041.