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Selection of peptides with surface affinity for α-chymotrypsin using a phage display library

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Abstract

Peptides with affinity for the surface of α-chymotrypsin (EC 3.4.21.1) were selected from a hexapeptide phage display library consisting of $\sim 10^7$ different clones. Seven selections were performed and five individual phage clones analysed. Compared to the primary library, the five peptide phage clones all interacted more strongly with α-chymotrypsin, and DNA sequencing of the phage clones revealed five different amino acid sequences: Gly-Ala-Val-Ile-Thr-His, Arg-Asp-Ile-Val-Val-Ala, Val-Tyr-Ser-His-Ala-Ser, Gly-Ser-Tyr-Ser-Ala-Gly and Leu-Asp-Ile-Val-Val-Ala. Two of the peptides exhibited 83% identity (i.e. a difference of just one amino acid). The chemically synthesized peptides competitively reduced the binding of the corresponding peptide phage clone to α-chymotrypsin. Binding of some of the selected peptide phage clones to α-chymotrypsin was also reduced by several of the other non-corresponding synthesized peptides, suggesting that these peptides have common recognition areas on the enzyme. Three of the synthesized peptides were poor substrates of α-chymotrypsin and they did not inhibit enzyme activity. Our results suggest that it is possible to select peptides from peptide phage display libraries with affinity for different surface structures on the enzyme, not involved in the biologically active site.

1. Introduction

The use of combinatorial phage libraries is a relatively new technique that has been widely used in several areas of biochemistry and biotechnology [1–4]. The technique makes it possible to screen simultaneously a massive number of protein-displaying phages, usually 10^7–10^8, for binding activities to a selector molecule. For instance, antibodies [5], hormones [6], inhibitors [7] and DNA-binding proteins [8–10] have all been expressed on phage coat proteins and subsequently engineered in order to increase or alter their affinity to a target molecule. Combinatorial libraries have, thus, in recent years provided a rational and effective way of engineering a protein without detailed knowledge of its molecular structure and contributed significantly to the understanding of interactions between molecules.

The combinatorial phage library technique was first introduced using phages expressing short peptides on the phage coat protein [11]. These epitope libraries were primarily used to find peptides with affinity for the biologically active

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sites of different selector molecules such as antibodies [12,13] and receptors [14,15]. However, libraries of linear peptides should also provide ligands capable of recognizing continuous epitopes outside the biologically active site of the selector molecule. Such peptide ligands should potentially be useful in the purification and isolation of biomolecules when applied, for instance, in the "weak affinity chromatography" mode [16]. Attempts to design peptides with affinity for the surface of a protein have been made with various success. For instance, detailed knowledge of the crystal structure of lysozyme led Atassi and Zablocki [17] to predict the amino acid sequences of putative affinity peptides to lysozyme. Short affinity peptides have also been designed using the information from crystal structures of the antibody–lysozyme complex [18].

In this paper we demonstrate that it is possible to use phage peptide libraries to select peptides with affinity for surface structures outside the biologically active site of a protein. Thus, five different hexapeptides have been selected, synthesized and characterized for their affinity to different surface areas on α-chymotrypsin.

2. Experimental[1]

2.1. Materials

The hexapeptide library [19] and *Escherichia coli* K91kan were a kind gift from Dr. George Smith (University of Missouri, Columbia, MO, USA). α-Chymotrypsin (EC 3.4.21.1) bovine pancreatic type II and trypsin–chymotrypsin inhibitor (Bowman-Birk) was from Sigma (St. Louis, MO, USA). HRP-labelled sheep anti-M13 immunoglobulin (IgG) and 2',2'-azino-bis(3-benzthiazoline-6-sulphonic acid) diammonium (ABTS) were from Detection Module Recombinant Phage Antibody System (Pharmacia, Uppsala, Sweden). Synthetic peptides were synthesized at Norsk Hydro Research Centre (Porsgrunn, Norway).

2.2. Covalent coupling of α-chymotrypsin to polystyrene petri dishes

Covalent coupling of α-chymotrypsin to polystyrene petri dishes was performed according to Chu and Tarcha [20] and modified to suit the conditions of immobilization in this study. Dry chlorosulphonic acid (1 ml, Janssen, Geel, Belgium) was allowed to react with polystyrene petri dishes (Falcon 3001, 3.5 cm diameter, Becton Dickinson, Plymouth, UK) for 20 min at room temperature on a shaker to activate the phenylic groups of polystyrene. The dishes were washed twice with cold nitromethane (Merck, Darmstadt, Germany) followed by the addition of 1 ml of diaminoethane (Merck) per dish which reacted with the activated phenylic groups to form spacers. The dishes were washed once with 1 ml of water, 2 ml of 1 *M* HCl, 1 ml of water, 2 ml of 1 *M* NaOH, 1 ml of water and 1 ml of methanol and dried under vacuum. A 750-μl volume of acetonitrile (Merck), 8 μl of bromoacetyl bromide (77 μmole (Merck) and 12 μl of triethylamine (77 μmole) (Merck) were mixed and added to each petri dish. The dishes were incubated for 2 h at room temperature on a shaker, washed three times with 1 ml of acetonitrile and dried under vacuum. A 100-μg amount of α-chymotrypsin in 50 m*M* Na_2HPO_4, 0.1 *M* NaCl, pH 7.4, containing 1 m*M* N-acetyl-D-tryptophan (Sigma), were incubated in each petri dish overnight at 4°C. The dishes were blocked with 1 ml of 5 mg/ml bovine serum albumin (BSA) in 0.1 *M* $NaHCO_3$ per dish for 1 h at 4°C on a shaker and washed six times with Tris-buffered saline (TBS) (50 m*M* Tris, 150 m*M* NaCl, pH 7.5), containing 0.5% (v/v) Tween 20 (Riedel-de Haën, Hannover, Germany).

2.3. Peptide synthesis and purification

9-Fluorenylmethoxy carbonyl (FMOC) chemistry and continuous-flow solid-phase peptide synthesis chemistry and (CFSPPS) [performed by a Biolynx 4170 upgraded to a NovaSyn

[1] Abbreviations for amino acids and peptides are according to the IUPAC-IUB Commission on Biochemical Nomenclature (IUPAC-IUB, 1984 and 1989).

Crystal peptide synthesizer (Novabiochem, Läufelfingen, Switzerland) were used to prepare the peptides [21]. Of each peptide $1 \cdot 10^{-4}$ mol were synthesized using four equivalents of 2-(1H-benzotriazole- yl)- 1,1,3,3-tetramethyluranium tetrafluoroborate (TBTU) (Novabiochem) activated FMOC amino acids in each coupling step [22]. Novasyn PA 500, with FMOC-Gly attached, was used as the solid support for the synthesis. The standard coupling and FMOC deprotection times were 45 and 7 min, respectively. Amino acid side-chain protection during the synthesis was as follows: Asp(OtBu), Arg(Pmc), His(Trt), Ser(tBu), Thr(tBu), Tyr(OtBu). FMOC removal was performed with 20% piperidine (Fluka, Buchs, Switzerland) in dimethylformamide (Fluka), and 95% aqueous trifluoroacetic acid (FFA) (Fluka) was used to cleave the peptides from the resin and for final removal of side-chain protection groups. The crude peptides were precipitated with diethyl ether, air-dried and lyophilized from 0.1% aqueous TFA.

The peptides were purified on a semipreparative reversed-phase (C_{18}) HPLC column eluted with a linear gradient of 0.1% TFA (aqueous) containing increasing acetonitrile concentrations using a Shimadzu LC8A chromatograph equipped with a SPD-6A UV–visible detector, a SIL-8A preparative autoinjector and a FCV-100B fraction collector (Shimadzu, Kyoto, Japan). The fractions were analysed by HPLC, and the selected fractions were combined and lyophilized.

2.4. Peptide analysis

Analytical reversed-phase HPLC was performed on a Nucleosil LC_{18} column (Mn 100-5, 250×4.6 mm I.D.) using a Shimadzu LC9A equipped with a SPD-6AV UV–visible detector and a SIL-9B autosampler. The detector was set to 214 nm, and samples were eluted with a linear gradient of 0.1% TFA (aqueous) containing increasing acetonitrile concentrations. Electrospray mass spectroscopy was performed on a Finnigan Mat SSQ710 (Finnigan Mat, San José, CA, USA), operating at 4 kV and 150°C. The drying gas (nitrogen) was applied at 25 ml/min, 0.2 MPa. Samples [0.1 mg/ml in 0.01% TFA (aqueous)–methanol (3:7)] and sheath liquid (2-methoxyethanol) were injected at 1 μl/min.

2.5. Selection procedure

Up to 10^{13} phage particles were incubated with 1–5 μg of α-chymotrypsin immobilized on petri dishes in 400 μl of TBS–Tween at 4°C on a rocking table overnight. The petri dish was washed with 1 ml of TBS–Tween ten times to eliminate non-specifically bound phages. Bound phages were eluted with 400 μl of 0.1 *M* HCl (the pH was adjusted to 2.2 with glycine) for 10 min on a shaker. The eluate was neutralized with 75 μl of 1 *M* Tris–HCl, pH 9.1 and concentrated on a Mikrosep microconcentrator, cut-off 10 kDa (Filtron Technology, Northborough, USA) after the first selection. It was not necessary to concentrate subsequent eluates. The eluates (100 μl) were amplified by infecting 100 μl of *E. coli* K91 cells in logarithmic phase for 20 min at room temperature and then cultivating in Luria-Bertani medium [23] overnight at 37°C on a shaker in the presence of 20 μg/ml tetracycline. The day thereafter, the *E. coli* cells were removed by centrifugation and the phages purified twice by precipitation with 0.15 volumes of polyethylene glycol (PEG)–NaCl [16.7%, w/v PEG, 3.3 *M* NaCl]. This selection procedure was repeated seven times with the input preparation in a selection being the amplified eluate of the preceding selection. Finally, the *E. coli* cells from the seventh eluate were plated out, and twenty-four single colonies were selected. The selected clones were grown and purified.

2.6. Characterization of α-chymotrypsin binding phage clones

α-Chymotrypsin (1 mg/ml) was incubated in phosphate-buffered saline (PBS) (140 m*M* NaCl, 25 m*M* KCl, 20 m*M* PO_4, pH 7.4) on a polystyrene microtiterplate (Maxisorp, Nunc, Roskilde, Denmark) (200 μl per well) overnight at 4°C. The wells were washed twice with PBS and blocked with 1% BSA (w/v) in PBS for 1 h at

37°C. The wells were washed twice with PBS followed by an addition of 10^{11} phage particles in PBS to each well, in triplicate, and incubated for 4 h at room temperature. The wells were washed twice with PBS, containing 0.05% (v/v) Tween 20 and twice with PBS. Horseradish peroxidase (HRP)-labelled sheep anti M13 IgG in PBS (1:5000), 200 μl per well, was added and incubated for 45 min at room temperature. Finally the microtiter plates were washed twice with PBS, containing 0.05% (v/v) Tween 20 and twice with PBS, followed by the addition of substrate (10 mg of ABTS, 45 ml of 50 m*M* citric acid, pH 4.0, 78 μl of 30% H_2O_2, 200 μl per well). Absorbance was monitored on a Multiscan MCC 340 (Labsystems, Helsinki, Finland) plate reader at 405 nm.

2.7. Competitive enzyme immunoassay (EIA)

The wells were treated as for the characterization of α-chymotrypsin-binding phage clones. Peptide 9 and peptide 24 were first dissolved in a small volume of dimethylsulfoxide before being diluted in PBS. The different peptides (30 μg/ml) were preincubated in PBS together with α-chymotrypsin, 1 h at room temperature. The different peptides were also present at the phage incubation step, at a concentration of 30 μg/ml.

2.8. Synthetic peptide digestion assay and α-chymotrypsin inhibition assay

Peptides 6, 17 and 21 (1 mg/ml) were digested by α-chymotrypsin (2.5 μg/ml) in Tris–HCl buffer, pH 7.6 (500 μl) at 25°C for approximately 24 h according to standard methodology [24]. Analytical reversed-phase high-performance liquid chromatography (HPLC) was performed on the digests [10 μg dissolved in 0.1% TFA (aqueous) as described previously. The fractions were then analysed with electrospray mass spectroscopy as described previously. A chymotrypsin inhibition assay was performed according to standard assay methodology [24] with α-chymotrypsin (2 μg/ml) and the synthetic peptides 6, 17 and 21 (1 and 2 mg/ml). Bowman-Birk inhibitor (10 μg/ml) was used as a control and inhibited α-chymotrypsin to 95%.

2.9. DNA sequencing

Single-stranded DNA from five individual phage clones was purified according to standard protocols using phenol and ether extraction and ethanol precipitation [23]. The DNA from the selected clones was amplified using polymerase chain reaction (PCR), with the PCR protocol and necessary reagents provided from the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The primer used for sequencing had the following nucleotide sequence: 5′-HO-CCCTCATAG-TTAGCGTAACG-OH-3′. The sequences were analysed on a 373A DNA sequencer (Applied Biosystems).

3. Results

3.1. Selection of phage peptides interacting with α-chymotrypsin

The hexapeptide phage library, consisting of approximately 10^7 individual clones, was selected seven times agains α-chymotrypsin. This resulted in an enrichment of phage clones that were able to interact with α-chymotrypsin. Table 1 shows that the yield of phages in the eluates from the different selections increased from 0.0000004% in the first selection to 0.5% in the last, which indicates that a selection has been accomplished. To enhance the selection, the input number of phage particles in selection number 5 was lowered [25] from 10^{13} to 10^{11}. Since BSA was used as a blocking agent in the immobilization procedure, an addition of BSA was made in the seventh selection to exclude possible BSA-binding phage clones.

3.2. Characterization of α-chymotrypsin interacting phage peptides

Twenty-four individual clones from the seventh preparation were tested for binding to

Table 1
Amplification protocol for the different selections using α-chymotrypsin as selector

Selection	Input preparation	Number of phage particles in input	Number of phage particles in eluate	Yield[a] (%)
1	Primary library	$2.3 \cdot 10^{12}$	$1.0 \cdot 10^{4}$	0.0000004
2	Eluate 1	$6.6 \cdot 10^{12}$	$1.9 \cdot 10^{8}$	0.003
3	Eluate 2	$1.3 \cdot 10^{13}$	$2.5 \cdot 10^{9}$	0.02
4	Eluate 3	$1.3 \cdot 10^{13}$	$2.7 \cdot 10^{9}$	0.02
5	Eluate 4	$1.3 \cdot 10^{11}$	$8.7 \cdot 10^{7}$	0.07
6	Eluate 5	$1.3 \cdot 10^{11}$	$6.3 \cdot 10^{8}$	0.5
7[b]	Eluate 6	$9.8 \cdot 10^{10}$	$4.9 \cdot 10^{8}$	0.5

[a] The yield was derived using the following formula: number of phage particles in eluate/number of phage particles in input × 100.
[b] The selection as performed with an addition of BSA in the phage incubation step.

α-chymotrypsin, using EIA methodology. The 24 individual clones were found to interact equally or more strongly with α-chymotrypsin compared to the primary library (data not shown). Five individual clones, showing the strongest interactions with α-chymotrypsin, were selected and analysed further. Peptide phage clones 7:6 and 7:21 displayed the strongest interaction with α-chymotrypsin and 7:24 the weakest interaction compared to the primary library (Fig. 1). DNA sequencing of the five individual phage genomes revealed five different amino acid sequences, which are shown in Table 2. Phage clones 7:9 and 7:24 showed 83% sequence identity (i.e. one amino acid was different).

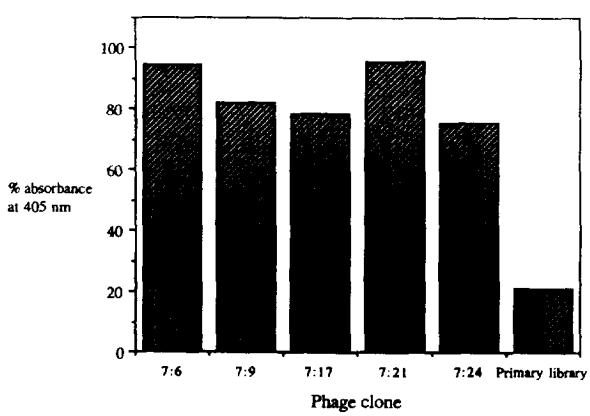

Fig. 1. Interaction of the five individual peptide phage clones with α-chymotrypsin compared to the primary library using EIA methodology. Mean values are based on thirteen different assays.

To further investigate the binding properties of the selected peptide phage clones the hexapeptides were chemically synthesized. Alanine and glycine amino acid residues, from the phage coat protein pIII, were added to the amino and carboxyl terminal ends to better mimic the hexapeptides on the phages. When the corresponding synthesized peptide and selected peptide phage clone were mixed in different ratios (e.g. peptide 7:6 and peptide phage clone 7:6), it was seen that the peptide could competitively inhibit binding of the peptide phage clone to α-chymotrypsin (Fig. 2a). The same behavior

Table 2
Amino acid sequences of the five selected hexamers

Phage clone	Amino acid sequence (X_6)
7:6	Gly-Ala-Val-Ile-Thr-His
7:9	Arg-Asp-Ile-Val-Val-Ala
7:17	Val-Tyr-Ser-His-Ala-Ser
7:21	Gly-Ser-Tyr-Ser-Ala-Gly
7:24	Leu-Asp-Ile-Val-Val-Ala
	1 2 3 4 11 12 13 14
pIII protein	NH_3-Ala-Asp-Gly-Ala-X_6-Gly-Thr-Ala-Gly-

Note: the selected amino acid sequences (X_6) are located after the fourth amino acid in the amino terminal of phage coat protein III (pIII).

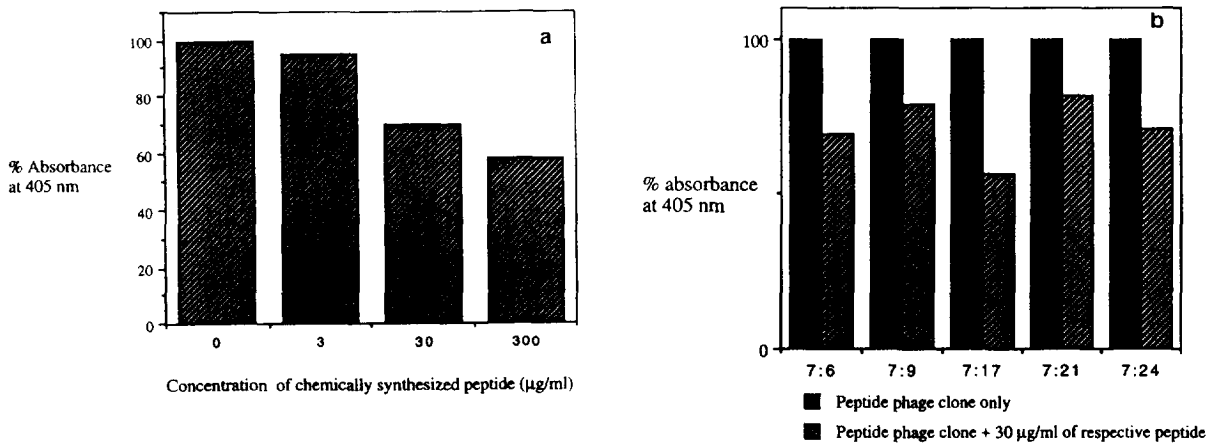

Fig. 2. (a) Peptide phage clone 7:6 binding to α-chymotrypsin in competition with different concentrations of peptide 6. Mean values are based on two different assays. (b) Binding of the different clones to α-chymotrypsin (black bars) and in competition with the corresponding synthetic peptides (hatched bars). Mean values are based on five to eight assays.

was seen when the other individual peptides were mixed with corresponding peptide phage clones (Fig. 2b).

Each individual peptide was also shown to reduce the binding of the other peptide phage clones to varying extents. Thus, the binding of peptide phage clone 7:17 to α-chymotrypsin was reduced by the corresponding peptide 17 and also by peptides 9, 21 and 24, but not peptide 6. The binding of peptide phage clone 7:21 was affected in a similar way, although not to the same extent, by peptides 9, 17 and 24. Peptides 6, 9 and 24, respectively, reduced the binding of the corresponding peptide phage clones to α-chymotrypsin to a higher degree than when the same concentration of the other peptides were added to the peptide phage clones (Fig. 3).

When peptides 6, 17 and 21 were digested by α-chymotrypsin over a period of 24 h (Table 3). peptides 17 and 21 were degraded into two smaller peptides. The cleavage sites were located between the tyrosine and serine amino acid residues, which is a known recognition site for α-chymotrypsin [26]. The degradation of peptide 6 was not complete and resulted in at least six different products. None of the three peptides had the ability to inhibit α-chymotrypsin, as judged from an activity assay for α-chymotrypsin.

Collectively, these results suggest that the selected peptides have affinity for different surface areas of α-chymotrypsin outside the biological active site.

4. Discussion

The results in this study suggest that it is possible to select peptides from a random hexapeptide phage library that interact with different surface areas outside the biologically active site of α-chymotrypsin. The hexapeptide library was selected seven times with α-chymotrypsin as a selector, which resulted in an enrichment of phage clones that were able to interact with α-chymotrypsin. Five individual α-chymotrypsin interacting phage clones from the seventh selection were further analysed and sequenced, and the five corresponding hexamers chemically synthesized. The five different peptides could competitively reduce the binding of the corresponding peptide phage clone to α-chymotrypsin and binding of some of the selected peptide phage clones were also reduced by non-corresponding synthetic peptides.

When the amino acid sequences of the five individual phage hexapeptides were investigated it was found that peptide 9 and peptide 24 (Arg-

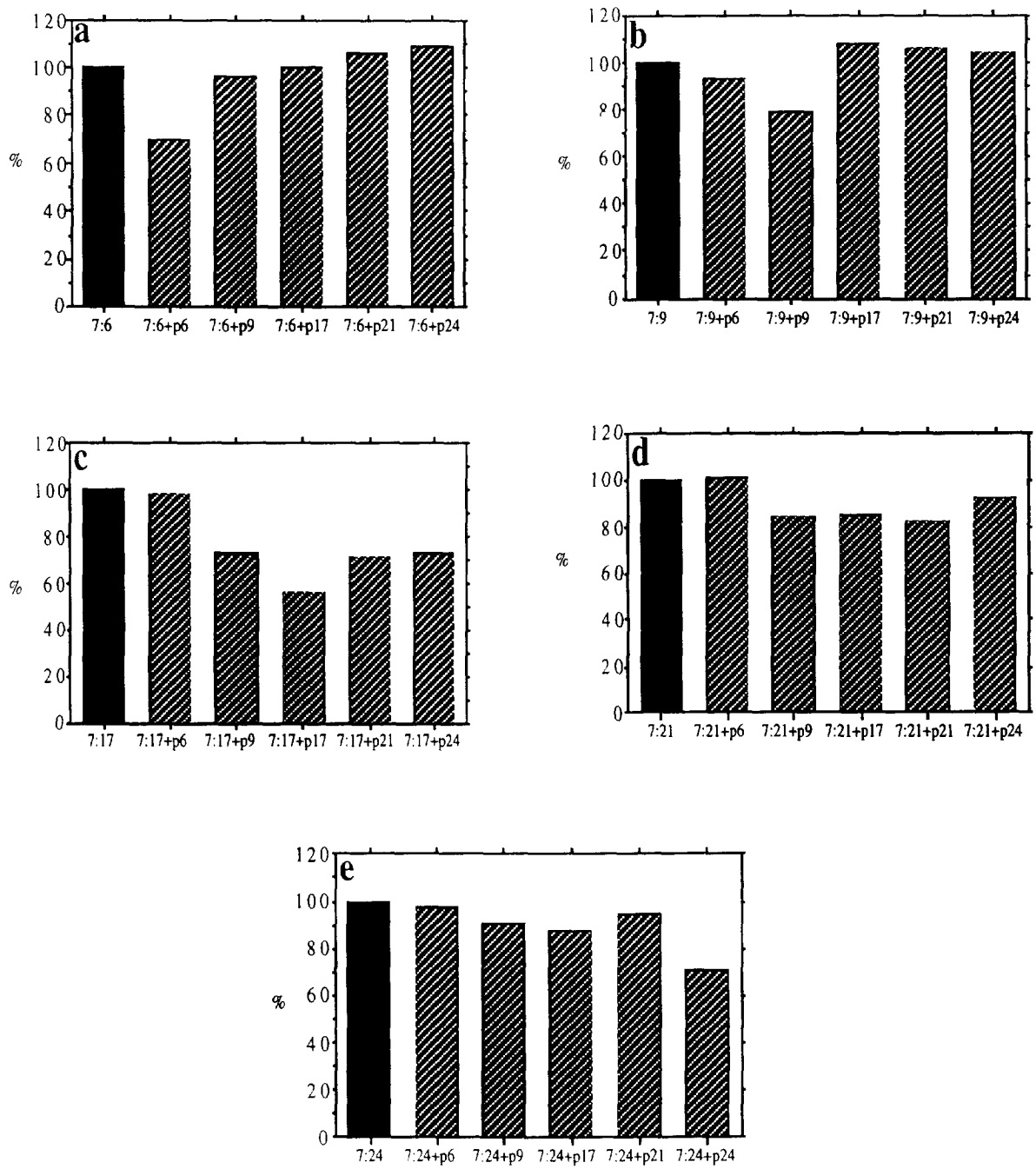

Fig. 3. Binding of the different peptide phage clones to α-chymotrypsin in competition with the different individual peptides. Black bars represent binding of respective peptide phage clone without competing synthetic peptide. (a) Phage clone 7:6, mean values from five different assays; (b) phage clone 7:9, mean values from six different assays; (c) phage clone 7:17, mean values from eight different assays; (d) phage clone 7:21, mean values from six different assays, (e) phage clone 7:24, mean values obtained from seven different EIA assays.

Table 3
Chymotrypsin digestion of peptide 6, peptide 17 and peptide 21

Peptide	Sequence	Peptide (HPLC) (%)		Degradation products
		0 h	24 h	
6	AGAVITHG	100	60	At least six different products
17	AVYSHASG	100	0	AVY, SHASG
21	AGSYSAGG	100	0	AGSY, SAGG

Asp-Ile-Val-Val-Ala and Leu-Asp-Ile-Val-Val-Ala), consisted of nearly identical residues, except for Arg and Leu in the first position. Thus, the hexamer in peptide phage clone 24 was almost entirely composed of hydrophobic residues. Crystallographic pictures of α-chymotrypsin show that hydrophobic areas are exposed on the enzyme surface, suggesting that these areas may be involved in the interactions with these peptides [27]. The similarity in amino acid sequence of peptide 9 and peptide 24 also suggests that both peptides interact with the same site on α-chymotrypsin. However, from the competitive EIA results (Fig. 3) it can be argued that the interaction sites on α-chymotrypsin are not identical since peptide 24 could not competitively inhibit the binding of peptide phage clone 7:9 and vice versa. One explanation for this could be that amino acid residues outside the hexapeptide on the phage coat protein are also involved in the interactions with α-chymotrypsin. These amino acid residues are not present on the chemically synthesized peptides since they consist only of the hexapeptide flanked by one amino acid of the phage coat protein III (pIII) on each side of the hexapeptide. Moreover, the synthesized peptides all have charged amino and carboxyl terminal residues, which may also affect the binding to the enzyme. Peptide 6 (Gly-Ala-Val-Ile-Thr-His) was the only peptide that could reduce the binding of peptide phage clone 7:6 and inhibited none of the other selected peptide phage clones. This suggests that this peptide binds to the surface of α-chymotrypsin outside the binding site(s) of the other peptides. The binding of peptide phage clone 7:17 was reduced by peptide 9, 17, 21 and 24, suggesting that these peptides interact with the same site as peptide 17, or with a site very close to or overlapping with this peptide. This is also supported by the fact that peptide phage clone 7:21 and 7:24 are inhibited by peptide 17. It is hard to explain why, for instance, peptide 17 cannot reduce the binding of peptide phage clone 9, as seen in Fig. 3b. Similar results have been obtained for peptide phage clone 7:9 and peptide 21. However, as mentioned above, the part of the selected peptide phage clone interacting with α-chymotrypsin may also include other amino acid residues outside the hexapeptide, thus making the results in Fig. 3 complex to fully interpret.

Cleavage studies over a period of 24 h showed that peptides 6, 17 and 21 acted only as weak substrates. Peptide 6 was not digested to completion and the digestion seemed to be non-specific since several minor degradation products were obtained. Peptide 9 and peptide 24 were not investigated due to their strong hydrophobicity, making the peptides insoluble in the recommended buffer at the necessary concentrations [24]. Neither of the peptides could inhibit the enzyme and investigations using Bowman-Birk inhibitor also confirmed that the active site is not involved in the binding process. Collectively, these results suggest that the interactions of the peptides with α-chymotrypsin take place on different surface areas of the enzyme not involved in the biologically active site.

When peptide phage clone 7:6 was allowed to compete for binding to α-chymotrypsin with different concentrations of peptide 6, an excess of the peptide was required to reduce the binding of the peptide phage clone. This suggests that the peptide phage clone interacts more strongly

with α-chymotrypsin than the corresponding peptide. Possible explanations for this could be that (i) the amino acid residues outside the hexamer (on pIII) contribute to the binding with the enzyme, as mentioned earlier. It is also possible that (ii) the hexamer peptides fused to the phage adopt a more preferable conformation for binding to α-chymotrypsin or (iii) that the close proximity of the peptides on the phage coat protein results in high local concentration of the peptides and a possible cooperativity in the binding to α-chymotrypsin, compared to the chemically synthesized peptides.

The results in this paper show that when a phage peptide library is screened against an enzyme, it is not obvious that the selected peptides interact only with the active site of the enzyme. Possible applications of short affinity peptides, as exemplified in this study, are as affinity ligands in weak affinity chromatography [16]. Separation systems based on weak affinity between the ligand and target molecule have been suggested to provide a valuable complement to traditional affinity chromatography, which often suffers from disadvantages such as high cost, low throughput and poor recovery of bioactivity. Weak affinity separation systems enable analytical investigations or preparative purification under mild buffer conditions with high recovery of bioactivity, and also favour rapid throughput and process scale-up. The use of small affinity ligands is also more beneficial than the use of specific antibodies with weak affinity to the target since the retention of a target molecule is dependent on a high concentration of active ligand coupled to the matrix. Small ligands with low molecular mass are therefore preferable. We are currently investigating the use of these peptides as affinity ligands in column chromatography [28].

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