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Some Recent Developments in the Preparation of Novel Recognition Systems: a Recognition Site for the Selective Catalysis of an Aldol Condensation using Molecular Imprinting and Specific Affinity Motifs for α-chymotrypsin using a Phage Display Peptide Library

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Molecular imprinting and phage display library technologies are rapidly being accepted as useful techniques for the generation of ligand-selective recognition motifs. The use of molecular imprinting to produce a novel type II aldolase mimic selective for the cobalt(II)-mediated aldol condensation of benzophenone and acetaldehyde is reported here. Furthermore, peptide motifs have been identified which are acting as 'affinity ligands' selective for the recognition of the enzyme α-chymotrypsin using phage display techniques.

Keywords: **molecular imprinting; phage display; synthetic enzyme; aldol condensation; α-chymotrypsin**

Introduction

Host-guest interactions are of paramount importance to biology. In recent years significant effort has been directed towards the development of systems mimicking those observed in nature, both with a view to furthering understanding of biology and for expanding upon the reportoire of interactions utilized by nature.

One such strategy is molecular imprinting (Andersson *et al.*, 1994; Ekberg and Mosbach, 1989; Mosbach, 1994; Shea, 1994; Wulff, 1993), a method for producing ligand-selective recognition sites in synthetic polymers. The technique has been shown to be useful in the preparation of artificial antibody combining site mimics (Andersson *et al.*, 1995a, b; Vlatakis *et al.*, 1993), with affinities and selectivities comparable to those of biologically derived antibodies, in the production of chiral chromatographic stationary phases (Fischer *et al.*, 1991; Nicholls *et al.*, 1995; O'Shannesy *et al.*, 1989; Wulff, 1993) and of potential for generating synthetic enzymes (Mallik *et al.*, 1994; Nicholls and Mosbach, 1993; Shea, 1994), in parallel with the development of catalytic antibody technology (Lerner *et al.*, 1991), albeit with modest success. The principles of molecularly imprinted polymer (MIP) preparation are summarized in Figure 1.

Another strategy for studying ligand/receptor interactions is the use of combinatorial libraries of biochemical or synthetic origin (Clackson and Wells, 1994, Houghten, 1994). With this technique it is possible to simultaneously screen a massive number of compounds against a target molecule. Peptide libraries expressed on phage coat proteins, with lengths from 5 to 20 amino acid residues, have been used to identify ligands with affinity for antibodies (Cwirla *et al.*, 1990), receptors (Koivunen *et al.*, 1993) and enzymes (Krook *et al.*,1995). Moreover, larger molecules such as antibody fragments (Winter *et al.*, 1994), hormones (Lowman *et al.*, 1991) and DNA binding proteins (Jamieson *et al.*, 1994; Krook, *et al.*, 1994) have been expressed on phage coat proteins and subsequently engineered in order to increase or alter their affinity to a selector molecule.

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Experimental

Molecular imprinting

DBM–Co^{2+} MIP preparation. Typical quantities: vinylpyridine (420 mg), styrene (4.20 g) and divinylbenzene (5.20 g) were mixed with the imprint molecule, dibenzoylmethane (DBM) (448 mg), initiator 2,2′-azobis(2,4-dimethyl valeronitrile) (100 mg) and cobalt(II) acetate (498 mg) in anhydrous methanol (2.5 ml) and chloroform (6.7 ml), briefly sonicated under vacuum then sparged with dry nitrogen (5 min) at 0°C. Polymerization was carried out at 45°C (24 h). The bulk polymer was ground in a mechanical mortar and wet sieved (water/ethanol) through a 25-μm sieve. The *fines* were removed by repeated sedimentation from acetonitrile and the sediment collected on a 15-μm filter. The print molecule complex (DBM–Co^{2+}) was removed by packing the polymer in an HPLC column and washing with methanol/acetic acid (7/3, v/v) for 24 h at 1.0 ml min^{-1}, then methanol. The reference

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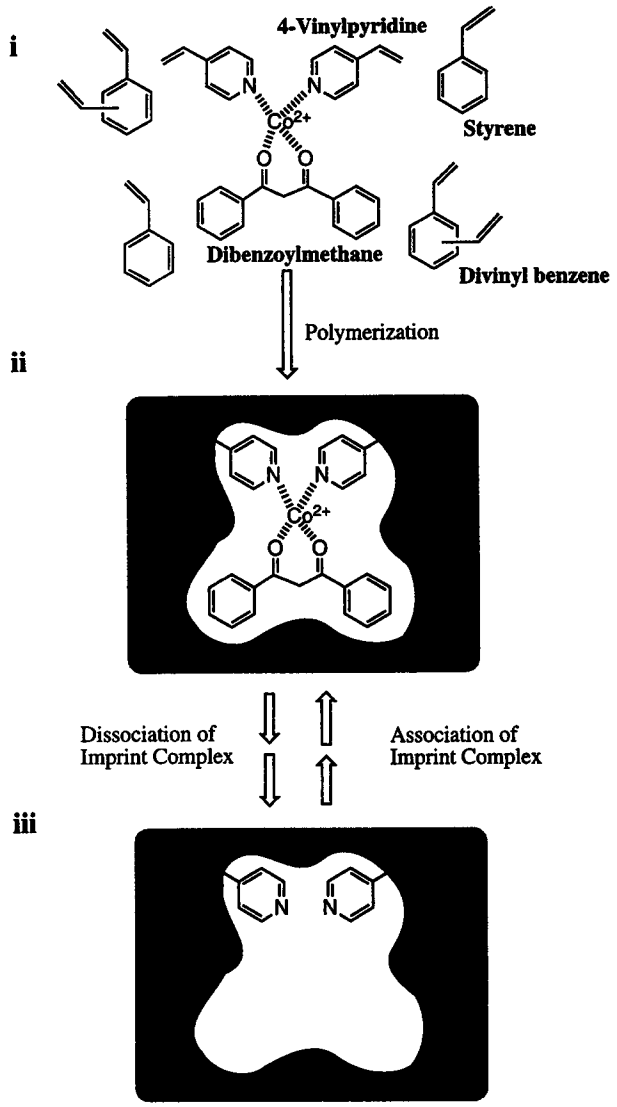

Figure 1. Schematic representation of MIP preparation: DBM–Co^{2+} MIP preparation (i) The monomers, vinylpyridine, styrene and divinylbenzene, are mixed with the imprint species, DBM and cobalt(II) acetate, and initiator in methanol, allowing formation of complementary sets of interactions between the monomers and the print species. (ii) Polymerization produces a rigid bulk polymer. (iii) The imprint species is extracted to render sites complementary to the imprint species in terms of shape and chemical functionality. (The schematic polymer structure depicted represents an idealized case and does not take into account the accessibility of the substrate into the recognition site.)

polymers were prepared and treated identically, except for the exclusion of one or both of DBM and cobalt(II) acetate.

MIP Assays. Polymer samples were typically incubated at room temperature for 12–16 h with cobalt(II) acetate [(10 mg per g polymer (dry weight)] in methanol (5.0 ml g polymer^{-1}). The polymers were collected on a 15-μm filter then dried under vacuum. [Co^{2+} determinations were conducted in duplicate on triplicate polymer samples. The Co^{2+} present in the polymers being calculated from the quantitative spectrophotometric (520 nm) determination of the residual Co^{2+} in the filtrate: DBM–Co^{2+} MIP: 5.76 and Co^{2+} MIP 5.44 mmol (site) g (polymer)$^{-1}$.] Co^{2+}-treated MIP samples (200 mg) were incubated with ketone (200 mmol) and benzaldehyde (200 mmol) in dry *N,N'*-dimethylformamide (1.0 ml). Solution reactions were carried out as described earlier with pyridine (0.01 ml) and cobalt(II) acetate (8 μmol). Sealed reaction mixtures were heated in a thermostatted oil bath at 100°C. Aliquots (10 μl) were taken directly from reaction mixtures, studies (data not shown) revealed no significant difference to results obtained by total extraction of the polymer matrix. Samples were analysed by RP-HPLC using a Serva C-18 column (300×2 mm) on an instrument comprising an LKB 2150 pump and LKB 2151 variable wavelength detector coupled to a Waters WISP 710B autoinjector (20 μl injection volume) and a Shimadzu C-R3A integrator recorder. Samples were serially diluted 100-fold in buffer before filtering and run isocratically using methanol/water (75:25) as eluent at 0.35 ml min^{-1}. Standard curves of concentration *versus* peak area and substrate/product peak ratios (not shown) were prepared over the concentration ranges used in the studies to allow yield calculation.

Phage display

Selection procedure. Up to 10^{13} phage particles were incubated with 1–5 μg α-chymotrypsin immobilized (Krook *et al.*, 1995) on Petri dishes (Falcon 3001, 3.5 cm diameter, Becton Dickinson, Plymouth, UK) in 400 μl tris-buffered saline (TBS)/Tween [50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.5 per cent (v/v) Tween 20] at 4°C on a rocking table overnight. The Petri dish was washed with 1 ml TBS/Tween 10 times to eliminate non-specifically bound phages. Bound phages were eluted with 400 μl 0.1 M HCl, pH adjusted to 2.2 with glycine, for 10 min on a shaker. The eluate was neutralized with 75 μl 1 M Tris-HCl, pH 9.1 and concentrated on a Mikrosep™ microconcentrator, cut-off 10 kDa (Filtron Technology Corporation, Nortborough, USA) after the first selection. It was not necessary to concentrate subsequent eluates. The eluates (100 μl) were amplified by infecting 100 μl of *Escherichia coli* K91 cells in logarithmic phase for 20 min at room temperature and then cultivated in Luria–Bertani medium overnight at 37°C on a shaker in the presence of 20 μg/ml tetracycline. The day after, the *E. coli* cells were removed by centrifugation and the phages purified twice by precipitation with 0.15 volumes polyethylene glycol (PEG)/NaCl [16.7 per cent (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 24 single colonies selected. The selected clones were grown and purified.

Characterization of α-chymotrypsin binding phage clones. α-Chymotrypsin (1 mg/ml) was incubated in phosphate-buffered saline (PBS) (140 mM NaCl, 25 mM KCl, 20 mM PO_4, pH 7.4), on a polystyrene microtitreplate (Maxisorp, Nunc, Roskilde, Denmark) (200 μl per well) overnight at 4°C. The wells were washed twice with PBS and blocked with 1 per cent bovine serum albumin (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 per cent (v/v) Tween 20 and twice with PBS. HRP-labelled sheep anti M13 immuloglobin G in PBS (1:5000), 200 μl/well, was added and incubated for 45 min at room temperature. Finally the microtitre plates were washed twice with PBS, containing 0.05 per cent (v/v) Tween 20 and twice with PBS, followed by the addition of substrate (10 mg 2′,2′-azino-bis(3-benzthiazoline-6-sulphonic acid) diammonium ABTS, 45 ml 50 mM citric acid, pH 4.0, 78 μl 30 per cent H_2O_2, 200 μl/well). Absorbance was monitored on a Multiscan MCC 340 (Labsystems, Helsinki, Finland) plate reader at 405 nm.

Competitive enzyme immunosorbent assay (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 dimethyl sulphoxide before dilution 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, in a concentration of 30 μg/ml.

DNA sequencing. Single stranded DNA from five individual phage clones was purified according to standard protocols using phenol and ether extraction and ethanol precipitation. The DNA from the selected clones was amplified using polymerase chain reaction (PCR), with PCR protocol and necessary reagents provided from the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The primer used for sequencing had the following nucleotide sequence: 5′HO-CCCTCATAGT-TAGCGTAACG-OH3′. The sequences were analysed on a 373A DNA Sequencer (Applied Biosystems, Foster City, USA).

Results

Molecular imprinting

A vinylpyridine–styrene–divinylbenzene copolymer was imprinted with a complex of DBM and Co^{2+}, a reactive intermediate analogue for the aldol condensation of acetophenone and benzaldehyde (Fig. 2). A series of reference polymers were similarly prepared imprinted with Co^{2+}, DBM, or nothing (detailed chromatographic analyses to be communicated elsewhere). Chromatographic retention characteristics of the reaction substrates, product and the imprint species were compared using a DBM–Co^{2+} imprinted polymer and the reference polymer systems. Reactions were conducted in the presence of the DBM–Co^{2+} and the Co^{2+} MIPs and in solution in the presence of pyridine and cobalt(II) ion (Fig. 3). The source of the observed catalytic activity was examined by using the transition state analogue, DBM, as an inhibitor (Fig. 4).

Phage display

The hexapeptide phage library was selected seven times against α-chymotrypsin. Individual phage clones from the seventh selection where picked and further investigated using EIA methodology (Fig. 5). Five individual peptide phage clones were found to interact more strongly with α-chymotrypsin than with the primary library (Fig. 6). DNA sequencing of the five individual phage genomes revealed five different amino acid sequences, of which two showed 83 per cent sequence identity (Table 1). To investigate further the binding properties of the selected peptide phage clones, the five peptides were chemically synthesised and the peptides and corresponding selected phages 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. 7).

Discussion

Molecular imprinting

The development of new methods for controlling the formation of carbon–carbon bonds is of significant interest. A MIP system utilizing the imprinting of a transition state analogue complex has been developed in an attempt to produce a class II aldolase mimic (Matsui *et al.*, 1996).

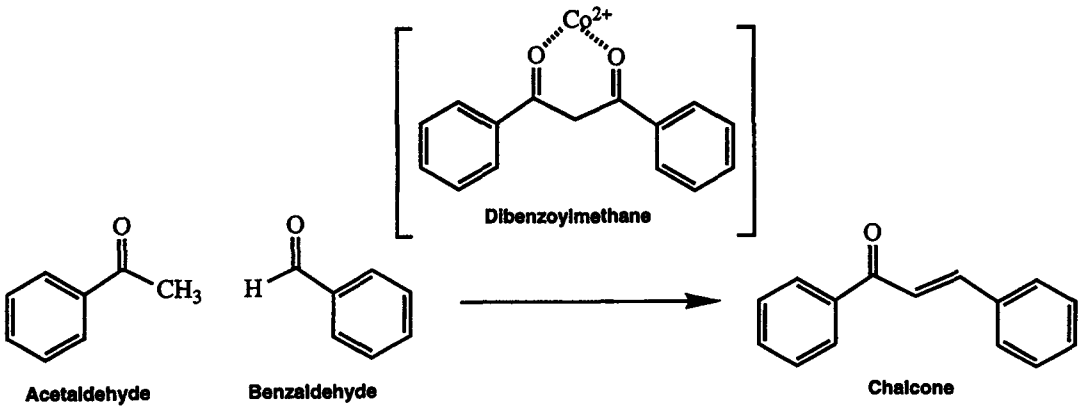

Figure 2. Aldol condensation of acetophenone and benzaldehyde to yield chalcone. The putative transition state analogue, DBM chelated to cobalt(II) ion, is shown above the reaction arrow.

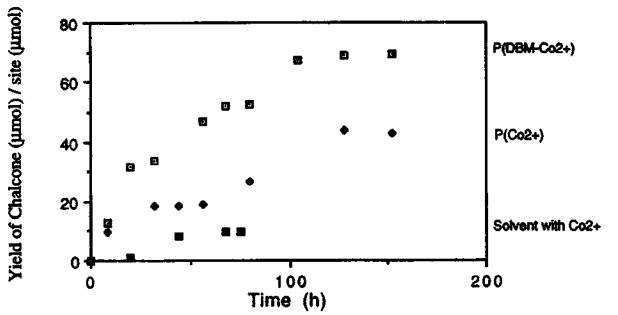

Figure 3. Production of chalcone using DBM–Co^{2+}, and Co^{2+} MIPs and solvent containing cobalt(II) acetate. Yields were calculated from the average of triplicate determinations of duplicate sets of reactions.

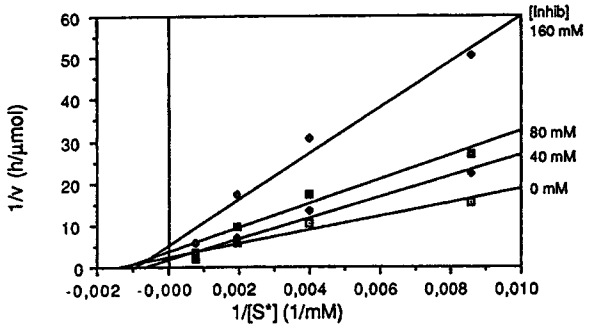

Figure 4. Lineweaver–Burk plot for the production of chalcone over a range of inhibitor concentrations [active site concentration 5.76 mmol g(polymer)$^{-1}$; maximum velocity $V_{max}=0.61\pm0.06$ mmol h^{-1}; Michaelis constant $K_m=1.23\pm0.04$ mM; $K_i=60\pm10$ mM].

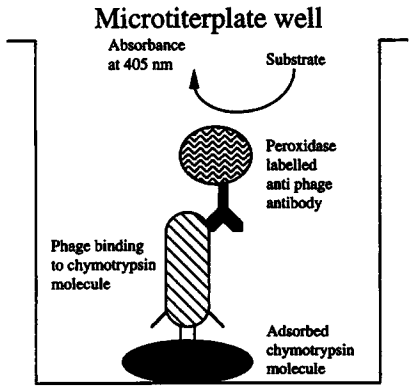

Figure 5. Schematic representation of the detection of chymotrypsin interacting phage peptide clones.

Table 1. 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 NH3-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).

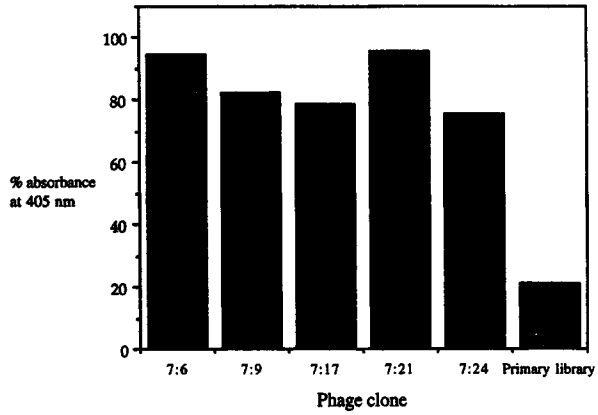

Figure 6. Interaction of the five individual peptide phage clones with α-chymotrypsin was compared with the primary library using EIA methodology. Mean values are based on 13 different assays.

HPLC studies employing the polymers as chromatographic stationary phases demonstrated that optimal recognition of DBM, the reactive intermediate analogue, was dependent upon both the presence of the coordinating metal and the complementary imprint site interactions. It is notable that the reactive intermediate analogue was the most efficiently recognized.

The DBM–Co^{2+} MIP-induced reaction rate, for the aldol condensation of acetophenone and benzaldehyde to produce chalcone, was eight-fold higher than for the corresponding

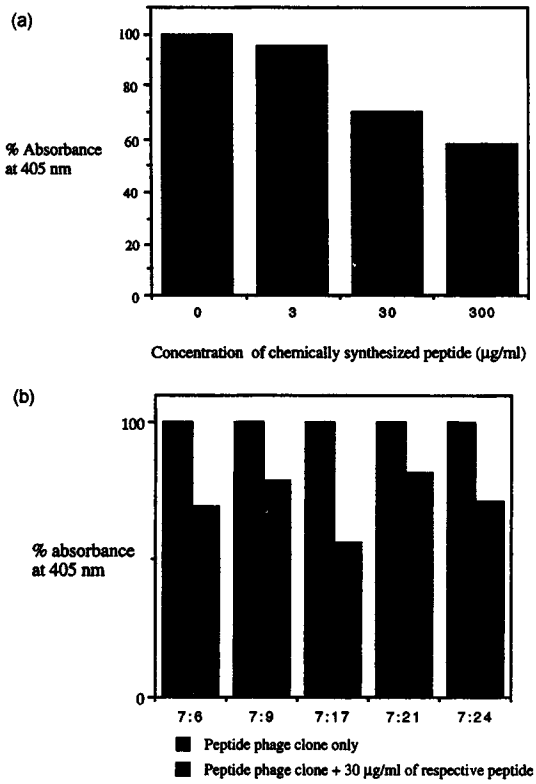

Figure 7. (a) Peptide phage clone 7:6 binding to α-chymotrypsin in competition with different concentrations of peptide 6. Mean values are based on two different assays. (b) Binding of the different clones to α-chymotrypsin (black bars) and in competition with the corresponding synthetic peptides (hatched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

solution reaction and twice that of the Co^{2+} MIP-mediated reaction (Fig. 3). This class II aldolase-like activity, as found in primitive cells such as yeast and bacteria, was competitively inhibited by DBM (Fig. 4). It is notable that the DBM–Co^{2+} MIP demonstrated turnover, nine-fold. This enzyme-like activity was achieved employing combinations of temperature (100°C), reaction time (days) and solvent (dimethylformamide) not possible when using biologically derived catalysts.

Phage display

The peptides corresponding to the variable domains of the selected phages competitively inhibited binding of the peptide phage clone to α-chymotrypsin (Fig. 7). Peptides 6, 17 and 21 did not have the ability to inhibit the enzymic activity of α-chymotrypsin, as judged using an activity assay for α-chymotrypsin. Peptides 9 and 24 could not be investigated due to their strong hydrophobicity, making the peptides insoluble in the recommended buffer at the necessary concentrations. Collectively, these results show that the selected peptides have affinity for surface areas on α-chymotrypsin outside the biological active site.

Conclusions

In summary, two recent developments in the area of artificial receptor preparation have been highlighted. Preliminary results from the authors' use of MIP-derived receptors (catalytic antibody mimics) capable of selectively catalysing the aldol condensation of acetophenone and benzaldehyde have been presented. The results demonstrate the use of molecular imprinting for preparing synthetic non-biological macromolecules capable of enzyme-like catalytic turnover, substrate selectivity and rate enhancement. Furthermore, this is the first report of true enzyme-like catalysis of carbon–carbon bond formation using MIPs.

Following the other approach presented, libraries of large numbers of different molecules are created, either biological or chemical, and used for identifying selective recognition features. In this brief account, peptide libraries were screened to identify peptide motifs selective for surface recognition of a protein, α-chymotrypsin. This methodology can be extended using larger molecules, e.g. antibody display libraries, for identifying larger receptors and enzyme mimics, e.g. catalytic antibodies. Both techniques are of great potential for the identification and production of recognition features for use in diagnostic and biotechnological applications.

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