Phage-Displayed Peptide Ligands for Pancreatic α-Amylase Cross-React with Barley α-amylase

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Peptide ligands that bind to pancreatic α-amylase were isolated from bacteriophage libraries displaying random 15-mer peptides by iterative affinity selection and amplification. The DNA sequences of selected clones from the final round of biopanning were determined. The two phage-display ligands with high-binding activities contained a high content of Arg, Tyr, and Trp residues with the short consensus sequence Arg-X-Tyr-Trp. These clones were shown to exhibit comparable binding interactions toward barley α-amylase based on transducing units titering and measurement of the dissociation constants.

Keywords: *Peptides; ligands; phage library; α-amylase*

INTRODUCTION

The phage-display technique provides a biological approach for creating diverse populations of peptide or protein variants by inserting random DNA coding sequences downstream of the signal sequence of gene *III* or *VIII* in filamentous bacteriophage (Smith, 1985). Peptides are expressed as N-terminal fusions to the coat proteins, and the resulting libraries are screened by panning the displaying particles against specific antigens for targeting activities/properties. The technique has been utilized for selecting biologically active peptides, engineering novel antibodies, isolating peptide mimetics and receptor ligands, identifying antigenic epitopes of proteins, and studying protein−DNA binding (Kay, 1994).

α-Amylases are endoglucosidases that cleave the α-1,4-glucosidic bonds in starch and other related oligo- and polysaccharides. All known α-amylases from various sources have the same basic $(\beta\alpha)_8$ barrel supersecondary structure (Jespersen et al., 1991), but only 20% of the amino acid residues in the catalytic domain of these enzymes are conserved (MacGregor, 1988). In a preliminary report, we described the use of combinatorial approaches to select peptide ligands for porcine pancreatic α-amylase (PPA) from a phage-displayed peptide library (Wong and Robertson, 1998). In this paper, we compare and confirm their cross-reactivity with barley α-amylase (BA) by determining the dissociation constants for the enzyme−ligand complex. The potential inhibitory effect of these ligands was also investigated.

MATERIALS AND METHODS

Enzymes and Reagents. Porcine pancreatic α-amylase from Boehringer Mannheim (Germany) was desalted and exchanged into TBS buffer. Barley α-amylase 1 was purified from crude α-amylase preparation purchased from Sigma (St. Louis, MO) according to the procedure described by MacGregor and Morgan (1992). Polyclonal antibodies for human pancreatic α-amylase were obtained from Accurate Chemical & Scientific Corporation (Westbury, NY). 2′,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was from Bio-Rad (Hercules, CA). Horseradish peroxidase-conjugated anti-phage antibody, consisting of horseradish peroxidase conjugated to monoclonal antibody against the bacteriophage M13 major coat protein product of gene *VIII*, was obtained from Pharmacia (Piscataway, NJ).

Affinity Selection of Peptide Ligands. The phage-display library was constructed by inserting degenerate oligonucleotides encoding all possible pentadecapeptides into the *Sfi*I site downstream of the signal sequence of gene *III* in phage vector fUSE5 (Scott and Smith, 1990). To isolate peptides ligands, the phage library was screened by affinity selection (Sparks et al., 1996). Each round of affinity purification started with immobilizing PPA (∼1.0 μg in 100 μL of 0.1 M $NaHCO_3$) on the surface of microtiter plate wells. After overnight incubation at 4 °C, the wells were blocked by adding 200 μL of blocking solution (5 mg/mL BSA in 0.1 M $NaHCO_3$). Following overnight incubation, the wells were washed with PBS−0.1% Tween 20 several times. Approximately 10^{11} pfu of the phage library was added. After 2 h incubation, the nonbinding phages were washed with PBS−Tween, and the bound phages were eluted by 50 mM glycine hydrochloride, pH 2.0. The process was repeated four times before individual phage clones were analyzed.

Putative binding phage clones obtained by panning were randomly picked and propagated. Binding activity was confirmed by ELISA according to Sparks et al. (1996). Enzyme-binding phage clones were detected using horseradish peroxidase-conjugated anti-phage antibody (Pharmacia, Piscataway, NJ). Bound-phage antibody carrying the peroxidase enzyme catalyzed the conversion of 2′,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) to a color product with an absorbance at 405 nm.

Measurement of Binding Activity. The phage clones thus identified were quantitatively characterized by transducing unit (TU) titering (Parmley and Smith, 1988). Bound-phage clones recovered in ELISA were used to infect *Escherichia coli* K91-Kan cells, followed by plating onto NZY plates supplemented with tetracycline and kanamycin (20 μg/mL). TU titers were estimated by counting colonies on these plates. Binding activity is defined as the percentage of input TU retained by the immobilized enzyme.

DNA Sequencing. Phage clones that showed positive binding activity as identified in the previous step were propagated, and phage ssDNA was purified using a QIAprep Spin M13 Kit (Qiagen, Santa Clarita, CA). DNA sequencing

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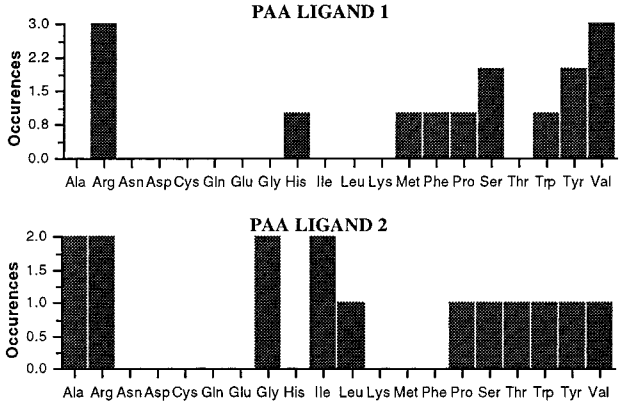

Figure 1. Histogram of amino acid composition of high-affinity phage-displayed peptides: clone 1 (RVVMSYPRHYWFSVR) and clone 2 (TRLGRIYWAAPSGIV).

was performed by the Sanger's dideoxy chain termination method using a Sequenase Kit (Amersham, Arlington Heights, IL).

Dissociation Constants of Enzyme−Ligand Complex. The dissociation constants of the two phage clones showing high-binding activity were measured based on the procedure described by Friguet et al. (1985). α-Amylase was equilibrated with a constant concentration of phage in PBS−Tween buffer for 2 days. The phage concentration was deduced from a preliminary ELISA calibration to give approximately half the maximum value in the ELISA assay. The free phage in the equilibrated mixture was determined by transferring 100 μL into the wells of a microtiter plate previously coated with the α-amylase. The bound phage were detected by adding anti-phage antibody conjugated with horseradish peroxidase and using ABTS for color development (Sparks et al., 1996). The dissociation constant was calculated from the slope of a Klotz plot (Klotz et al., 1946).

Inhibition Assay. Serial dilutions of phage-displayed peptides were preincubated with an equal volume of appropriately diluted α-amylase. The enzyme reaction was initiated by adding 300 μL of 1% soluble starch. After incubation at 25 °C for 10 min, aliquots of 100 μL were removed and added to 0.5 mL of copper−bicinochoninate reagent and 0.4 mL of water (Waffenschmidt and Jaenicke, 1987). The mixture was heated in a water bath at 80 °C for 30 min and cooled for 15 min, and the absorbance was measured at 560 nm.

RESULTS AND DISCUSSION

The library was screened in two separate experiments for clones that bind to PPA. In a preliminary report (Wong and Robertson, 1998), 18 out of 80 clones screened by ELISA showed an absorbance greater than 0.55, and the amino acid sequences of the binding peptides in these clones (designated 1−5) were deduced from their respective DNA sequences: RVVMSYPRHYWFSVR, TRLGRIYWAAPSGIV, ASRHAIRFIVFPAT, LPLVFLTCLIMLSRV, VSAAPTPAYWFGFYY. For a quantitative analysis of phage binding, the percentage of input TU retained by the immobilized target α-amylase and the percent recovery from the binding reaction were calculated. Binding activities of 18.5, 12.6, 6.8, 3.6, and 2.8% were obtained for clones 1−5, respectively. All phage clones also retained comparable activity toward BA1 based on the analysis by TU titering. Clones 1, 2, and 5 phage-displayed peptides had high p*I* values between 11 to 13 and a net charge between +2 to +3 at pH 7.0. The high positive net charges suggest the importance of Arg residues, which have been known to be involved in salt bridge-type interactions in the

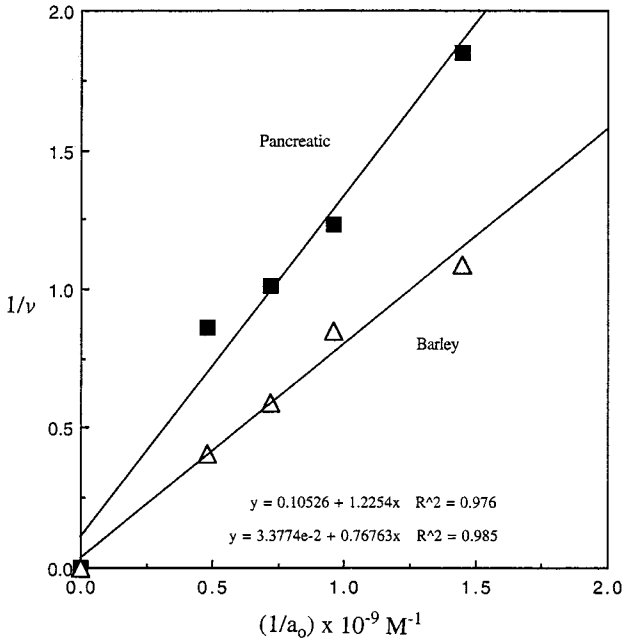

Figure 2. Klotz plots of the binding of clone 1 phage-displayed peptides to PAA and BA1.

complex formation between pancreatic α-amylase and red kidney bean α-amylase inhibitor. Mutation Arg19 → Leu in the α-amylase inhibitor, Tendamistat, causes a 100-fold decrease in the binding affinity (O'Connell et al., 1994). A similar pattern in the amino acid composition was observed in the tight-binding peptide ligands identified for BA1 in our previous investigation (Wong et al., 1998). The tight binding phage-displayed peptides identified for BA1 contained Trp, Tyr, and Phe spaced in between Arg residues. One of the peptides identified contained a Tyr-Arg-Trp triplet similar to the conserved segment found in all common α-amylase inhibitors. These tight binding peptides also had calculated p*I* values ≥10, with a net charge of +2 or higher at pH 7.0.

The clone that showed the highest affinity for PAA was further characterized by measuring the dissociation constant using an indirect competition method described by Friguet et al. (1985). The dissociation constants of clone 1 phage-displayed peptide binding to pancreatic and barley enzymes are 8.2×10^{-10} and 1.3×10^{-9}, respectively (Figure 2). These results substantiate the TU titering experiment, which indicates that the peptide ligands cross-react with the barley enzyme with considerable binding affinity. The K_d values obtained are within the range of those reported for natural α-amylase inhibitors. Tanizaki and Lajolo (1985) obtained K_d values of 1.7×10^{-10} and 4.4×10^{-9} M at pH 5.5 and pH 6.9, respectively, for the enzyme−inhibitor complex at 37 °C. Powers and Whitaker (1977) reported a K_d of 3.5×10^{-11} M (pH 6.9, 30 °C) for porcine pancreatic α-amylase and red kidney bean amylase inhibitor. The clone with the highest binding affinity to BA1 enzyme had a K_d of 4.4×10^{-9} M for the displayed peptide−enzyme complex (Wong et al., 1998). The K_i of Tendamistat against PAA is about 9×10^{-12} M (Vertesy et al., 1984). BA2 and BASI associate tightly at pH 8 with a K_d of 2.2×10^{-10} M (Abe et al., 1993). The direct use of recombinant phage for functional assays has been reported in the selection of peptide epitopes (Willis et al., 1993) and in the screening of peptide inhibitors for trypsin (Fang et al., 1996).

These results are in contrast to the binding properties of the BA1 peptide ligands, which showed 1/2−1/5 less activity for PAA (Wong et al., 1998). Apparently, the peptide ligands for PPA exhibited less specificity as compared to the BA1 ligands. It has been postulated that α-amylases from barley and porcine pancreas contain nine subsites A−I and five subsites D−H, respectively (Ajandouz et al., 1992; Seigner et al., 1987). The substrate binding site of the pancreatic enzyme consists of a less number of subsites than barley isozyme and, hence, exhibits less flexibility in accommodating conformational changes during ligand binding. MacGregor and MacGregor (1985) suggested that small variations in the loops and helices forming the subsites of different amylases may account for the changes in the binding specificity of the enzymes.

The two highest binding peptides (1 and 2) shared as a common feature a high number of Arg, polar (Tyr, Trp), and hydrophobic (Val, Ala, Phe, Pro) residues (Figure 1). Clones 1 and 2 contained a consensus sequence of Arg-X-Tyr-Trp. Clones 1 and 5 contained Pro-XX-Tyr-Trp-Phe. These results are reminiscent of the highly conserved triad found in all known pancreatic α-amylase inhibitors that bind to the enzyme active site (Vertesy and Tripier, 1985). High-resolution X-ray structure analyses of porcine pancreatic α-amylase in complex with Tendamistat indicate that majority of the interactions involve Arg and Tyr residues located in four binding segments of the inhibitor (Wiegand et al., 1995). The crystal and NMR structures of tendamistat-type inhibitors suggest that the triad residues are located in a type I β-turn on the protein surface (Kline et al., 1988; Billeter et al., 1989). The lectin-like protein α-amylase inhibitor from common bean also contains a similar motif for binding interactions (Mirkov et al., 1995). A three-dimensional structure of PAA and the lectin like inhibitor suggests that residues Trp188, Arg74, and Tyr190 closely match the tripeptide sequence in bacterial α-amylase inhibitor, but only Tyr190 forms a strong interaction with the catalytic Asp residue with two other Tyr residues (37 and 186) bound to the other catalytic residues (Bompard-Gilles et al., 1996). The barley α-amylase/subtilisin inhibitor (BASI) that is active on barley α-amylase 2 but not on isozyme 1 involves the binding of Arg155, which is essential for inhibition (Rodenburg et al., 1995). A recent report on the crystal structure of BA2−BASI complex clearly suggests that $Arg128_{BA2}$ undergoes a conformational stabilization on binding to BASI (Vallee et al., 1998).

A preliminary experiment was performed to investigate if the phage-displayed peptides are inhibitors of amylases. The results indicate that the binding of these phage-displayed ligands to the two amylases did not show an inhibitory effect on the enzyme activity with soluble starch as the substrate. Although it is likely that the binding occurs at sites other than the active site, the possibility of interactions at the substrate binding site without interference with the catalytic activity cannot be ruled out entirely. It should also be noted that the present work could potentially lead to displayed peptide ligands that interact with other binding sites in PPA and not necessarily to the active site or are inhibitory. In addition to the active site that is located in a cleft between the C-terminal side of the $(\alpha/\beta)_8$ barrel of domain A and domain B, a second binding site has been identified on the surface of PPA that may play a role in anchoring long-chain starch substrates (Buisson et al., 1987). A maltose binding site has been identified at the interface between domains A and C and shown to bind a maltoside unit in oligosaccharide substrates (Qian et al., 1997). A binding site for calcium ion has been identified as essential for structural stabilization, and a chloride binding site is located near the C-terminal end of the β barrel of domain A (Qian et al., 1993, 1994). The data obtained in the present investigation are insufficient to suggest the actual binding site of the identified peptide ligands. Computer modeling should provide a better insight into the interactions between these ligands and the enzyme. The binding and/or inhibitory activities could be further confirmed and characterized by investigating the effects of mutagenesis of the sequences using synthetic peptides.

CONCLUSION

This study demonstrates that identification of peptide ligands for PPA could be achieved by iterative screening and selection of phage-display libraries without prior knowledge of the structure−function properties of these peptides. The high-affinity PPA peptide ligands were shown to contain a common sequence of Arg-X-Tyr-Trp and to interact with the structurally homologous barley enzyme.

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