Screening of an α-Amylase Inhibitor Peptide by Photolinker–Peptide Array

Takashi Ochiai, Tomoya Sugita, Ryuji Kato, Mina Okochi, and Hiroyuki Honda

Department of Biotechnology, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8603, Japan

Received December 15, 2011; Accepted January 14, 2012; Online Publication, April 7, 2012

Peptide arrays in which peptides were immobilized on cellulose membranes through photolinkers were synthesized. The peptides were subsequently detached from the arrays by ultraviolet (UV) photolysis for 3 h, and were used to search for functional peptides that inhibit the activity of α-amylase derived from human pancreatic juice. Amino acid replacement with high-molecular-size amino acids, Arg (R), Phe (F), Trp (W), or Tyr (Y), for the first and seventh residues of amylase inhibitor peptide, GHWYYRCW, as previously reported, led to enhancement of the inhibitory effect of the peptide on α-amylase. In particular, one of the resulting peptides, RHWWYRYW, showed a stronger inhibitory effect than acarbose (which is used as a hypoglycemic agent) or inhibitor peptide GHWYYRCW.

Key words: amylase inhibitors; combinatorial screening; hypoglycemics; peptide arrays; photolinker

Peptides consist of short sequences (up to about 12 monomer units) of the 20 or so naturally occurring amino acids linked together in various combinations through peptide bonds. Although they have lower molecular weights than proteins, peptides act as signaling molecules in the control of many physiological actions in vivo. For example, an active peptide domain has been reported, and there are many reports on functional peptides from relatively long-chain peptides, e.g., defenses and nisin, which show antibacterial action, a peptide vaccine that works by inducing cytotoxic T lymphocytes, and cell-penetrating peptides isolated from human immunodeficiency virus (HIV), to short-chain peptides, RGD (Arg-Gly-Asp), GLP (Gly-Leu-Pro), HIRL (His-Ile-Arg-Leu), and lactotripeptide, VPP (Val-Pro-Pro) and IPP (Ile-Pro-Pro).

It has been estimated that 246 million people around the world suffer from diabetes, and in most developed countries, this disease is linked to several major causes of death. Improvements in lifestyle can be effective against postprandial hyperglycemia, but a failure to ameliorate postprandial hyperglycemia by dietary and exercise therapies can result in a need for medication. Generally, therapeutic agents aimed at ameliorating postprandial hyperglycemia of Type 2 diabetes inhibit enzymes such as α-amylase or α-glucosidase, which are involved in the digestion and assimilation of carbohydrates in the intestinal tract, thereby suppressing the rapid elevation of blood glucose levels after meals. Acarbose, a tetrasaccharide, is a typical oral hypoglycemic agent used in treating Type 2 diabetes that inhibits the activity of α-glucosidase and α-amylase by binding to the active sites of these enzymes.

Proteins derived from various plants, particularly cereals such as soybean, rice, and wheat, can also act as α-amylase inhibitors. In addition, Dolecková-Maresová et al. have reported that the linear peptide GHWYYRCW is an inhibitor of α-amylase. This octamer peptide was designed by means of combinatorial chemistry as a small-sized inhibitor of α-amylase. Various other inhibitors of α-amylase have been developed in this manner, but there is still a need to develop α-amylase inhibitors that show no side effects and are useful in preventing or treating diabetes, obesity, and postprandial hyperglycemia.

A peptide array is a tool for analyzing peptides that is produced by means of the peptide spot synthesis, developed by Frank and co-workers. Using a peptide synthesizer, 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids are spotted at arbitrary sites on the cellulose membranes and subsequently converted into a peptide chain through elongation by repeated solid-phase synthesis. Such peptide arrays are widely used for various interaction analyses. In particular, they are used as epitope-mapping tools, protein-interaction domains, kinases, receptors, and cytokines.

Using peptide arrays, we have succeeded in finding various functional peptides that bind to various receptors, including hypotensive peptides, cell death-inducing peptides, cell-adhesion peptides, nanoparticles-recognizing peptides, and bile acid-binding peptides. In peptide arrays, the peptides are immobilized on cellulose membranes. To find peptides that interact with the active sites of enzymes, the peptides must be detached from the cellulose membranes to permit them to interact directly with the enzymes.

Various attempts have been made to use photocleavage in peptide synthesis, and various photolinkers have been developed. Of these photolinkers, we chose 4-[4-(1-[[1H-fluoren-9-ylmethoxy]carbonyl]amino]ethyl)-2-methoxy-5-nitrophenoxyl]butanoic acid (compound no. 26 reported by Holmes), which allows peptides to be rapidly detached from the solid phase by irradiation with UV at 365 nm.

---

1 To whom correspondence should be addressed. Fax: +81-52-789-3214; E-mail: honda@nubio.nagoya-u.ac.jp

Abbreviations: DIPC, N,N-diisopropylcarbodiimide; DMF, N,N-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; MOE, Molecular Operating Environment; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid
In this study, we synthesized peptide arrays in which the peptides were linked through the above-mentioned UV-cleavable photolinker, and searched for functional peptides that inhibit the activity of pancreatic α-amylase. We used peptide GHWWYRCW as our initial sequence, and searched for sequences that have a greater inhibitory effect than GHWWYRCW, with the aim of discovering new peptide inhibitors of α-amylase.

**Materials and Methods**

**Synthesis of the photolinker.** The photolinker used in this study was synthesized in six steps from 1-(4-hydroxy-3-methoxyphenyl)ethanone (acetovanillone, H0261; Tokyo Chemical Industry, Tokyo). Acelavinolane (5.50 g, 0.03 mol), methyl 4-bromobutyrate (3.77 mL, 0.03 mol), and K2CO3 (6.22 g, 0.045 mol) were dissolved in N,N-dimethylformamide (DMF; 25 mL), and the solution was stirred for 16 h at room temperature to give methyl 4-(4-acetyl-2-methoxy-xyphenoxy)butanoate. The product was dissolved in 2.1 mixture of pyridine and H2O (26.25 mL), and then H2SO4-HC1 (2.51 g, 0.03 mol) was added and the mixture was stirred for 14 h at room temperature to give methyl 4-[4-(N-hydroxyethanamido)2-methoxy-xyphenoxy]butanoate. To a solution of this product in glacial acetic acid (79.07 mL) was added 10% palladium on charcoal (0.494 g). This mixture was stirred overnight under a hydrogen atmosphere (3 atm). Pyridine (59.3 mL) and TFA (6.16 mL, 0.044 mol) were added and the mixture was stirred for 1 h to give methyl 4-[2-methoxy-4-[1-(fluoroxy)ethyl]-2-methoxy-xyphenoxy]butanoate. The product (6.22 g, 0.045 mol) was mixed with 70% HNO3 (136 mL) at 0 °C, and water was added to bring the final volume of the solution to 1,361 mL. The mixture was stirred overnight to complete nitration, and then filtered. The resulting solid product was washed and crystallized from MeOH/H2O to give methyl 4-[2-methoxy-5-nitro-4-[1-(fluoroxy)ethyl]-2-methoxy-xyphenoxy]butanoate. MeOH (94.9 mL) and 1 m aqueous NaOH (38.0 mL) were added to the product (6.16 g, 0.044 mol), and the solution was stirred for 14 h. The resulting product was treated with FmocCl (3.8 g, 0.014 mol) in 1,4-dioxane, and the pH of the solution was adjusted to 8 with 1 m triethylamine. The mixture was left to stand for 30 min. 1 m aqueous HCl (100 mL) was added, and the solution was diluted to a final volume of 1 L with water. The resulting mixture was added to stand for 18 h to obtain a crystallized product, 4-[4-1-([(1H-fluoren-9-ylmethyl)carbonyl]amino)ethyl]-2-methoxy-5-nitrophenoxy]butanoic acid, and this (3.8 g, 0.0073 mol) was stored at 4 °C in darkness.

**Synthesis of peptide arrays.** A cellulose membrane (grade 542; Whatman, Maidstone, UK) was activated using β-alanine as the N-terminal basal spacer. A photolinker, 4-[4-[1-([(1H-fluoren-9-ylmethyl)carbonyl]amino)ethyl]-2-methoxy-5-nitrophenoxy]butanoic acid (compound no. 26 reported by Holmes et al.,) was developed as linker for Fmoc peptide synthesis. This was used in the following strategy. The photolinker conjugated as a photolinker between the candidate peptides and the cellulose, Activated Fmoc amino acids (0.5 mm) were spotted to the membrane using a peptide auto-spotter (ASP222; Intavis, Cologne, Germany) following the manufacturer’s instructions, with some modifications. After first residue addition, the remaining amino groups were blocked with 2% acetic anhydride. At each elongation step, the membrane was deprotected with 20% piperidine and then washed thoroughly with N,N-dimethylformamide, followed by methanol. After final deprotection, side-chain protecting groups were removed for 3 h with a mixture of TFA (A00025; Watanabe), m-cresol (034-04646; Wako), ethane-1,2-dithiol (A00057; Watanabe), and (methyl-sulfanyl)benzene (T0191; Tokyo Chemical Industry) at a ratio of 40:1:3:6. Finally, the membrane was washed thoroughly with diethyl ether, methanol, and phosphate-buffered saline (PBS; pH 7.4), consecutively, and dried under sterile conditions in preparation for the remaining assay procedures. Three spots for each peptide sequence were deposited on each membrane.

**Release of peptides from the photolinker–peptide arrays.** Holmes reported that 4-[4-1-([(1H-fluoren-9-ylmethyl)carbonyl]amino)ethyl]-2-methoxy-5-nitrophenoxy]butanoic acid, selected by us, allows peptides to be detached from the solid phase by irradiation with UV at 365 nm. Hence, the following strategy for release of peptide was carried out: When the photolinker–peptide array had dried completely at room temperature, it was irradiated with UV at 365 nm (T-15L UV tube; 15 W, 365 nm) for 3 h using a transilluminator (DT-20LCP; Atto, Tokyo). After irradiation, each spot on the array was punched out to a 6-mm in diameter using a biopsy punch (KAI Corp., Gifu). Each of the resulting peptide-containing disks was placed in a well of a 96-well plate, and 100 μL of PBS (pH 6.9) was added. Then the plate was centrifuged at 1,000 rpm for 5 min to give a supernatant solution containing the soluble peptide.

To measure the amount of peptide released from each of the peptide-containing disks, we quantified free peptides using phthalaldehyde (AnaLyte®OPA Protein Quantitation Kit; AnaSpec, Fremont, CA). First, a photolinker–peptide array with glycine trimer (GGG) was synthesized, and the resulting peptides were solubilized by subjecting them to UV irradiation for various durations. The phthalaldehyde solution used was prepared by adding 2-mercaptoethanol (24 μL) as a reducing agent to phthalaldehyde (60 μL) and diluting the mixture to a total volume of 2 mL with PBS. The phthalaldehyde solution (20 μL/well) was added to the solution of the soluble peptide (100 μL/well), and the fluorescence intensity was measured by means of a plate reader (Fluoroskan Ascent LS L210470; Labsystems, Waltham, MA) with excitation at 355 nm and recording of the emissions at 460 nm.

**Enzyme-inhibition experiments.** In the enzyme-inhibition experiments, the inhibitory effects of the inhibitor peptides were estimated by competitive experiments involving enzymes, substrates, and inhibitor peptides. An Ultra Amylase Assay Kit (E33651, EnzChek®; Life Technologies Japan, Tokyo) was used in the enzyme-inhibition experiments. DQ starch, a cornstarch derivative labeled with BODIPY FL dye and supplied in the assay kit, was used as substrate, and was dissolved in PBS (pH 6.9) to a final concentration of 1 mg/mL. For the enzyme, α-amylase derived from human pancreatic juice (203A0050; Calzyme, San Luis Obispo, CA) was dissolved in PBS (pH 6.9) at a concentration of 0.1 μg/mL. The human pancreatic α-amylase solution (50 μL/well) and the solutions of soluble peptides released from the photolinker–peptide array (100 μL/well) were added to a 96-well plate and kept at 37 °C for 1 h. The DQ starch (100 μg/mL, 50 μL/well) substrate was then added, and the plate was incubated at 37 °C for 1 h. When the reaction was complete, the fluorescence intensity released from the DQ starch was measured using a fluorescence plate reader (Fluoroskan Ascent LS L210470) with excitation at 485.0 nm and recording of emissions at 505.0 nm.

Enzyme activity was assayed by estimating the amount of fluorescent substance released from fluorescent substrate BODIPY FL in comparison with a calibration curve. One unit (U) of enzyme activity was defined as the potency required to release 1 μmol of the BODIPY FL dye in 1 min. The molar concentration of the DQ starch [mm] was defined as amount of BODIPY FL dye in a DQ starch molecule, which can be released by amylase digestion.

**Steric interactions between the enzyme and peptides.** The steric interactions between the active sites of the α-amylase and the peptides that inhibited the activity of α-amylase, as identified by the screening process, were analyzed by means of a docking simulation in the Molecular Operating Environment (MOE; Ryoka Systems, Tokyo). Data on the active sites of human pancreatic juice-derived α-amylase (PDB 1HNY) were used in this simulation.

**Results**

**Analysis of free peptides released from the photolinker–peptide array**

After the photolinker-peptide array was irradiated with UV, the amount of peptide released from the peptide array was analyzed by the OPA method. When we synthesized glycine trimer (GGG) on the photolinker–peptide array, the amount of free peptide was approximately 15 nmol, and this amount came to be constant after 3 h of UV irradiation. It has been reported
that a peptide array affords 28 nmol/spot (0.28 cm²) of peptides. The amount of peptides released in this experiment thus corresponded to 54% of the value reported in the literature.

When we synthesized an actual α-amylase inhibitor peptide (sequence, GHWYYRCW; molecular weight, 1,170) on the photolinker–peptide array and released it by UV irradiation, subsequent analysis by mass spectrometry (MS) (Autoflex II (TOF/TOF); Bruker Daltonics, Billerica, MA) indicated that the molecular weight of the soluble peptide obtained was 1,170. Furthermore, the sequence was determined to be GHWYYRCW (Gly-His-Trp-Tyr-Tyr-Arg-Cys-Trp) by MS/MS analysis. Thus we confirmed that peptides immobilized on the photolinker–peptide array could be released by UV irradiation over 3 h to give the corresponding soluble peptides.

**Enzyme-inhibition experiments with photolinker–peptide arrays**

We synthesized a photolinker–peptide array containing the glycine octamer (GGGGGGGG) and peptide sequence GHWYYRCW, which has been reported to inhibit amylase activity. Two peptides (GHWYYRCW and GGGGGGGG) without photolinkers were also synthesized on the peptide array as negative control. Figure 1 shows the results of the enzyme-inhibition experiments using the products of irradiation of the peptide arrays containing GHWYYRCW and GGGGGGGG with and without photolinkers.

Only GHWYYRCW with the photolinker showed an inhibitory effect (27%) on the enzyme activity of human pancreatic α-amylase. GHWYYRCW without the photolinker (the negative control) showed no inhibitory effect, because the peptide was not released by UV irradiation. The glycine octamer also showed no inhibitory effect. These results confirm that a peptide with an inhibitory effect on α-amylase inhibited the activity of the enzyme on being released from the photolinker–peptide array by UV irradiation. They also indicate that the peptide immobilized on the peptide array was not suitable for assay of enzyme-inhibitory activity.

![Fig. 1. Amylase Activity by Peptide Array with Photolinker.](image1)

**Amylase Activity by Peptide Array with Photolinker.**

Peptide was synthesized with and without photolinker on cellulose membrane. After the peptide array was irradiated with UV at 365 nm, each spot was punched out to 6 mm in diameter and placed in a well of a 96-well plate. Human pancreatic α-amylase solution (50 μL/well) and DQ starch (100 μg/mL, 50 μL/well) substrate were added to above plate and incubated at 37°C for 1 h as described in “Materials and Methods.”

**Search for α-amylase inhibitor peptide**

Using our photolinker–peptide arrays, we searched for improved peptides that strongly inhibit the activity of human pancreatic α-amylase. First, by substituting glycine for each of the amino acid residues in the sequence of α-amylase inhibitor peptide GHWYYRCW, we identified the residues that are important in inhibiting the activity of α-amylase (Fig. 2).

When we replaced the second, third, fourth, sixth, or eighth residue with glycine, the inhibitory effect of the GHWYYRCW analogues on the activity of human pancreatic α-amylase was reduced. When the fifth or the seventh residue was replaced with glycine, however, the inhibitory effect on the activity of α-amylase was enhanced. This suggests that if the fifth or the seventh residue is replaced by another of the 20 natural α-amino acids, a more strongly inhibiting peptide might be produced. Because GHWYYRGW, in which the seventh residue was replaced, showed a marked inhibitory effect on the activity of α-amylase, we examined further modifications of GHWYYRGW in our screening studies from this point onward.

In our second round of screening, we examined sequences in which each residue in GHWYYRGW was replaced by an amino acid other than glycine and determined the inhibitory effects of the resulting peptides on the activity of α-amylase. In this screening, the second, third, fourth, sixth, and eighth residues were replaced by amino acids having similar properties in terms of hydrophobicity, molecular weight, and isoelectric point (pI). For this purpose, we selected 13 amino acids, Arg (R), Gln (Q), His (H), Ile (I), Leu (L), Lys (K), Met (M), Phe (F), Pro (P), Ser (S), Trp (W), Tyr (Y), and Val (V), for use in the experiments. The first, fifth, and seventh residues were similarly replaced by each of the 19 amino acids in turn for screening.

The results are shown in Fig. 3. When we replaced the first and seventh residues with R, F, W, or Y and the second residue with R, the inhibitory effect on the activity of α-amylase was improved as compared with that of the reference sequence (GHWYYRGW). R, F, W, and Y have higher molecular weights than the 16 other natural amino acids, and F, W, and Y contains an aromatic ring each. In other words, amino acids that have a high molecular weight and contain an aromatic ring are important in the interaction of
peptides with the active site of human pancreatic juice-derived α-amylase.

In our third screening, we used peptide sequences in which the first, second, and seventh residues were replaced by one of four amino acids (R, F, W, or Y), and we examined the inhibitory activity of the resulting peptides on α-amylase (Table 1).

We identified 18 peptides that inhibited the activity of α-amylase by more than 60%. In particular, when both the first and the seventh residue were replaced by R, F, W, or Y, there was a tendency for the peptides to have a strong inhibitory effect on the enzyme activity. To inhibit the activity of α-amylase, the peptides must adopt a conformation in which they can interact better with the active site of the enzyme. We hypothesized that this is assisted by the presence of amino acids with high molecular weights that contain an aromatic ring, such as R, F, W, or Y. Of the sequences that inhibited the activity of α-amylase obtained in our third screening, we selected double-substitution product RHWYYRYW (Arg-His-Trp-Tyr-Tyr-Arg-Tyr-Trp) for subsequent experiments.

Inhibitory effects of an α-amylase-inhibitor peptide

We compared the inhibitory effect and the inhibitory mechanism of RHWYYRYW, a peptide obtained in our third screening, with those of GHWYYRCW and acarbose, which is used as a hypoglycemic agent and inhibits the enzyme activity of α-glucosidase and α-amylase. The powdered peptides were produced by custom manufacturing (Invitrogen), and we conducted enzyme-inhibition experiments for various concentrations of the inhibitor peptide and the substrate.

RHWYYRYW, GHWYYRCW, and acarbose all inhibited the activity of α-amylase, as shown in Fig. 4. We found that RHWYYRYW had a stronger inhibitory effect than acarbose or GHWYYRCW. The IC_{50} of the amylase activity in the case of 50 mg/L of initial substrate concentration was determined to be 6 µM for RHWYYRYW, while 25 µM and 19 µM were obtained...
Amylase Inhibition with Peptides GHWYYRCW (Fig. 4).

Table 1. Results of Third Screening

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Sequence</th>
<th>Amylase activity (× 10^{-4} U)</th>
<th>Peptide no.</th>
<th>Sequence</th>
<th>Amylase activity (× 10^{-4} U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RYWYRRYW</td>
<td>3.07</td>
<td>9</td>
<td>YRWYRFW</td>
<td>3.29</td>
</tr>
<tr>
<td>2</td>
<td>YYWYRRYW</td>
<td>3.07</td>
<td>10</td>
<td>RYWYRRW</td>
<td>3.30</td>
</tr>
<tr>
<td>3</td>
<td>RRWYRRYW</td>
<td>3.13</td>
<td>11</td>
<td>FRWYRRW</td>
<td>3.31</td>
</tr>
<tr>
<td>4</td>
<td>YRWYRYRW</td>
<td>3.13</td>
<td>12</td>
<td>FRWYRYW</td>
<td>3.36</td>
</tr>
<tr>
<td>5</td>
<td>WWYYRRRW</td>
<td>3.19</td>
<td>13</td>
<td>WRWYRRW</td>
<td>3.38</td>
</tr>
<tr>
<td>6</td>
<td>YKWYRRRW</td>
<td>3.25</td>
<td>14</td>
<td>RWYRYRW</td>
<td>3.40</td>
</tr>
<tr>
<td>7</td>
<td>WRWYRRRW</td>
<td>3.25</td>
<td>15</td>
<td>WRYYRW</td>
<td>3.40</td>
</tr>
<tr>
<td>8</td>
<td>RHWYRRW</td>
<td>3.28</td>
<td>16</td>
<td>WYWYRW</td>
<td>3.40</td>
</tr>
<tr>
<td>9</td>
<td>YRWYRW</td>
<td>3.29</td>
<td>17</td>
<td>RWYRW</td>
<td>3.41</td>
</tr>
<tr>
<td>10</td>
<td>FYWYRW</td>
<td>3.44</td>
<td>18</td>
<td>FYWYRW</td>
<td>3.44</td>
</tr>
<tr>
<td>11</td>
<td>YKWYRW</td>
<td>3.55</td>
<td>19</td>
<td>YKWYRW</td>
<td>3.55</td>
</tr>
<tr>
<td>20</td>
<td>RHWYRW</td>
<td>3.61</td>
<td>21</td>
<td>RWYWRF</td>
<td>3.61</td>
</tr>
<tr>
<td>22</td>
<td>RWYWRF</td>
<td>3.62</td>
<td>23</td>
<td>WRWYRF</td>
<td>3.62</td>
</tr>
<tr>
<td>24</td>
<td>GRWYRW</td>
<td>3.67</td>
<td>25</td>
<td>RYWYRW</td>
<td>3.68</td>
</tr>
<tr>
<td>26</td>
<td>FHWYRW</td>
<td>3.72</td>
<td>27</td>
<td>FRWYRF</td>
<td>3.75</td>
</tr>
<tr>
<td>28</td>
<td>RYWYRW</td>
<td>3.76</td>
<td>29</td>
<td>RWYRW</td>
<td>3.77</td>
</tr>
<tr>
<td>30</td>
<td>FRWYRGW</td>
<td>3.77</td>
<td>31</td>
<td>WRWYRGW</td>
<td>3.78</td>
</tr>
<tr>
<td>32</td>
<td>WRWYRGW</td>
<td>3.78</td>
<td>33</td>
<td>YHWYRGW</td>
<td>3.78</td>
</tr>
<tr>
<td>34</td>
<td>FRWYRW</td>
<td>3.94</td>
<td>35</td>
<td>FRWYRGW</td>
<td>3.95</td>
</tr>
<tr>
<td>36</td>
<td>YHWYRGW</td>
<td>4.05</td>
<td>37</td>
<td>GHWYRGW</td>
<td>4.05</td>
</tr>
<tr>
<td>38</td>
<td>GWYYRW</td>
<td>4.30</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Inhibition of Amylase Derived from Other Sources by RHWYYRYW

<table>
<thead>
<tr>
<th>Inhibitor concentration, I (µM)</th>
<th>Amylase activity (mU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse pancreatic</td>
</tr>
<tr>
<td></td>
<td>α-amylase</td>
</tr>
<tr>
<td>0</td>
<td>3.9 × 10^{-3}</td>
</tr>
<tr>
<td>8.75</td>
<td>5.6 × 10^{-4}</td>
</tr>
<tr>
<td>35</td>
<td>5.2 × 10^{-4}</td>
</tr>
<tr>
<td>140</td>
<td>3.2 × 10^{-4}</td>
</tr>
</tbody>
</table>

for GHWYYRCW and acarbose respectively. When we examined the inhibitory effect of RHWYYRYW on α-amylases derived from human saliva (A1031; Sigma-Aldrich, St. Louis, MO) and α-amylase derived from mouse pancreatic juice (MA103; Elastin Products, Owensville, MO), RHWYYRYW also inhibited the activity of these enzymes (Table 2).

*Fig. 4. Amylase Inhibition with Peptides GHWYYRCW (●), RHWYYRYW (○), and Acarbose (▲) under Initial Substrate Concentrations of 50 mg/L (a) and 100 mg/L (b).*

**Discussion**

In this study we synthesized peptide arrays in which the peptides were immobilized on a cellulose membrane through photolabile linking groups. The group of synthetic soluble peptides detached from the membrane by photocleavage was searched for functional peptides that inhibit the enzyme activity of α-amylase derived from human pancreatic juice.

First, we synthesized photolinker 4-[4-(1-[(1H-fluoren-9-ylmethoxy)carbonyl]amino)ethyl]-2-methoxy-5-nitrophenoxyl]butanoic acid, and linked it with peptides synthesized on the peptide arrays. The immobilized peptides in the peptide array were released by UV photolysis over 3 h for subsequent use in enzyme-inhibition assays.

By using this system and by taking as a starting peptide GHWYYRCW, previously reported to inhibit human pancreatic α-amylase, we were able to screen a series of peptides and to identify those that showed stronger inhibitory activity than our selected starting peptide. Substitution of high-molecular-weight amino acids (R, F, W, and Y) for the first and seventh residues of the base peptide, GHWYYRCW, markedly enhanced the inhibitory effect of the resulting peptide on the activity of the enzyme.

As a second screening, we performed a comprehensive series of amino acid replacements to search for peptides...
that inhibit α-amylase. We identified sequences that showed more than twice the inhibitory effect of a positive control. These inhibited more than 60% of the activity of α-amylase. In addition, this screening identified the sequence WYYR (Trp-Tyr-Tyr-Arg) in the peptides as a sequence important to inhibitory activity.

In general, peptides containing D-amino acids have longer half-lives in vivo and are less readily degraded than those containing the corresponding natural L-amino acids. Hence, we substituted D-amino acids into the peptides to stabilize them in vivo and evaluated the amylase inhibition activity of the resulting analogues (Ochiai et al., unpublished results). We examined the sequences in which every residue of RHWYYRW, identified in the third screening, was replaced by the corresponding D-amino acid. The sequences in which all eight residues were replaced by D-amino acids showed a 35% decrease in inhibitory effect. Since 65% of the inhibitory effect remained, the D-amino acid analogue was considered to be a useful inhibitory peptide for in vivo use. In addition, sequences in which the third, fourth, fifth, and sixth residues (WYYR) were replaced by the corresponding D-amino acids showed lower inhibitory effects than the D-amino acid analogue sequences in the first, second, seventh, and eighth residues. These results suggest that the three-dimensional structure of WYYR is important for interaction with the active site of human pancreatic juice-derived α-amylase. Docking simulations using MOE suggested that WYYR interacts with and fits in the active site of α-amylase to inhibit the enzyme’s activity.

Comparisons using powder peptides produced by a custom manufacturing service (Invitrogen) showed that RHWWYRYW inhibited α-amylase better than GHWWYRCW or acarbose, which is used as a hypoglycemic agent.

Acknowledgments

We thank Professor Nobuhiwa Watanabe of the Graduate School of Engineering, Nagoya University, for his advice and great help in the docking simulation and Professor Akira Sakakura of the Graduate School of Engineering, Nagoya University, for his advice and great help in the synthesis of the photolinker.

References