**Alteration of substrate specificity of rat neurolysin from matrix metalloproteinase-2/9-type to -3-type specificity by comprehensive mutation**

Tetsuya Kadonosono, Michiko Kato-Murai and Mitsuyoshi Ueda

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo, Kyoto 606-8502, Japan

1To whom correspondence should be addressed. E-mail: miueda@kais.kyoto-u.ac.jp

The substrate specificity of rat brain neurolysin was rapidly modified by semirational mutagenesis coupled with a yeast molecular display system. Neurolysin mainly recognizes substrates with sequential six residues close to the scissile bond in polypeptides, cleaving a peptide bond in the center position of the six residues. To alter the recognition of the P2′ amino acid of substrates by neurolysin, six residues of neurolysin, Asp467, Arg470, Glu510, Tyr606, Tyr610 and Tyr611, which might be involved in the formation of the neurolysin S2′ subsite, were individually and comprehensively substituted. The protein libraries of mutant neurolysins comprising 120 species were displayed on the yeast cell surface and screening was carried out using two fluorescence-quenching peptides, the matrix metalloproteinase-2/9-(MMPs-2/9)- and MMP-3-specific substrates, which consisted of similar amino acids, except for alanine (for MMPs-2/9) or glutamic acid (for MMP-3) at the P2′ amino acid position. Among mutant neurolysins, the Y610L mutant neurolysin exhibited a marked change in substrate specificity. Steady-state kinetic analysis of the purified Y610L mutant neurolysin revealed that the binding efficiency towards the MMP-3-specific substrate was about 3-fold higher than that toward the MMP-2/9-specific substrate. These results indicate that Tyr610 of neurolysin is the important residue to recognize the P2′ amino acid of substrates.

**Keywords:** matrix metalloproteinase/molecular display/neurolysin/peptidase/substrate specificity

---

**Introduction**

Peptidases catalyze the hydrolysis of peptide bonds, which includes processing of bioactive peptides to regulate signal transduction, length regulation of antigenic peptides for the antigen presentation of major histocompatibility molecules and cleavage of peptides for the recycling system of amino acids. Substrate specificity and recognition residues of peptidases have been extensively investigated to clarify the natural substrates and the role of peptidases and to understand the structure–function relationship of proteins (Xu and Li, 2005; Graham et al., 2006; Ito et al., 2006; Diamond, 2007; Gass and Khosla, 2007). Modification of the substrate specificity of peptidases using protein engineering technology is a powerful strategy to gain the basic knowledge of peptide recognition. However, there are few studies on the alteration of substrate specificity of peptidases. Bacterial protease OmpT is a successful example of the alteration using a protein-display system (Varadarajan et al., 2005).

The construction of a gene library and its conversion to a protein library following screening for function has progressed with the development of several protein-display systems in protein engineering (Boder and Wittrup, 1997; Ueda and Tanaka, 2000; Benhar, 2001; Frankel et al., 2003; Daugherty, 2007; Seeling and Szostak, 2007). Currently, the protein library is constructed on the basis of a semirational strategy as follows (Antikainen et al., 2003; Geddie and Matsumura, 2004; Rui et al., 2004; Schmitzer et al., 2004; Shiraga et al., 2005; Fukuda et al., 2007). Multiple and specific residues are selected on the basis of structural and functional knowledge, then DNA codons encoding the selected residues are comprehensively changed using mixed primers (Chica et al., 2005). The focused library designed by the semirational approach has decreased library size and provided a higher possibility for acquiring positive clones than in the case of a freely randomized library constructed by the directed evolution approach including the error-prone PCR- and DNA-shuffling methods.

Conventional phage and bacterial displays are powerful screening methods for libraries of mutant proteins, because proteins displayed on the cell surface can be easily and rapidly assayed without purification steps (Benhar, 2001; Daugherty, 2007). These display systems cannot, however, display proteins having large molecular masses or post-translational modifications. Yeast display is one alternative method for producing a large amount of proteins in their active form (Ueda and Tanaka, 2000). Because yeast can produce large proteins having post-translational modification accompanied by protein-folding control, the yeast display system has become a novel and comprehensive protein producer and an attractive tool for screening methods, including the direct sequence determination of selected clones by DNA sequence analysis.

In this study, the substrate specificity of a rat brain neuropeptidase, neurolysin, was modified by the semirational protein engineering method to clarify the substrate recognition mechanism of neurolysin. The crystallographic structure of neurolysin has been resolved, and its narrow pocket is involved in substrate recognition (Brown et al., 2001). Neurolysin mainly recognizes substrate peptides with six-amino-acid-long sequences with the recognition motif PXX↓X-gallery-XX (motif 1) or XX↓X-XX (motif 2), where the letters indicate following: P, proline residue; X, aromatic, basic or hydrophobic residues; X-gallery- aromatic or large hydrophobic residues; X-Basic residues; and ↓, cleavage position (amino acid from the N-terminus of substrates were denoted P3, P2, P1, P1′, P2′ and P3′, and the corresponding binding spaces in neurolysin were numbered S3, S2, S1, S1′, S2′ and...
S3' subsites, in accordance with the standard nomenclature (Schechter and Berger, 1967; Kadonosono et al., 2007b). In previous research on the substrate specificity of neurolysin (Kadonosono et al., 2007a), neurolysin effectively cleaves the matrix metalloproteinase-2/9 (MMPs-2/9)-specific peptide MOCAc-RPKPYANvawMK(Dnp)-NH2 at the peptide bond between proline and tyrosine, whereas it cleaves the MMP-3-specific peptide MOCAc-RPKPEvawWRK(Dnp)-NH2 at the peptide bond between proline and valine with the about 1/10 efficiency compared with the cleavage of the MMPs-2/9-specific peptide, where MOCAc, Dnp and Nva represent the (7-methoxy-coumarin-4-yl) acetyl group, 2,4-dinitrophenyl group and norvaline, respectively. These amino acid sequences were compared with those of recognition motif 1, the MMPs-2/9-specific peptide exactly matched; however, the MMP-3-specific peptide had an unfavorable glutamic acid residue at the P2' amino acid position, suggesting that glutamic acid in the MMP-3-specific peptide reduced its recognition by neurolysin. Therefore, for the recognition of P2' amino acids of substrates, the S2' subsite of neurolysin was modified to realize the binding of neurolysin to glutamic acid by semirational mutagenesis of the residues involving in the formation of neurolysin S2' subsite. We measured the activity of neurolysin towards the MMP-3-specific peptide using the yeast molecular display platform. The substrate recognition mechanism on the S2' subsite of neurolysin will be discussed below.

Materials and methods

Strains and media

Escherichia coli DH5α [F-, φ 80dlacZ ΔM15, Δ (lacZYA-argF)U169, hsdR17 (rK-mK), recA1, endA1, deoR, thi-1, supE44, gyrA96, relA1, λ-] (Toyobo, Osaka, Japan) was used as a host for DNA manipulation. Saccharomyces cerevisiae strain B12168 (MATa, leu2, trp1, ura3-52, prb1-1122, pep4-3, prcl-407, gal2), a protease-deficient strain, was used as a host for protein production. E. coli transformants were grown in Luria–Bertani (LB) medium [1% (w/v) tryptone, 0.5% yeast extract and 1% sodium chloride] containing 50 μg/ml ampicillin. Yeast host cells were grown in SDC+UW medium (0.7% yeast nitrogen base without amino acids, 2% glucose, 1.5% casamino acids with 0.002% uracil and 0.002% tryptophan) and yeast transformants were cultivated in SDC+W medium (0.7% yeast nitrogen base without amino acids, 2% glucose, 1.5% casamino acids with 0.002% tryptophan) or SGC+W medium (0.7% yeast nitrogen base without amino acids, 2% galactose, 1.5% casamino acids with 0.002% tryptophan).

Construction of plasmids

A DNA fragment encoding rat brain neurolysin was inserted into the pGEM vector (Promega, WI, USA) (Kadonosono et al., 2007b). The fragment containing the neurolysin-encoding region was digested by SacII and XhoI, then ligated into the SacII–XhoI sites of pLyc-Lc (Okochi et al., 2007). In the ligated, linker and FLAG tag sequence (amino acid sequence: GSAGSGYDDDDKGSAGSG) were inserted between neurolysin and α-agglutinin regions using two primers, 5'-CGAAGCGCTCCTGGTTCGTCGTCG-3' and 5'-GATAAGAGCTTTTGGCGCTCCATCTCCCTCTCTCTCTCTCTCTAC-3' and

![Fig. 1](image-url)
CAACAAATGCTTNNKAACCTGGGTGTGGGAC-3' and 5'-G TCCACACCCAGTNNMAAGCATTTGGATGAGG-3' for E510X (pDAU-NF-E510X), 5'-CCTGGAGCATGTCCATGCCGTC-3' and 5'-ATATTGGGCCATCMNCC CCTCTGCCAAATG-3' for Y606X (pDAU-NF-Y606X), 5'-TATGGATGCGCAANNTATGGATATTGTG-3' and 5'-CCAAAGATCATCTAMTNNTTGCCATCATA-3' for Y610X (pDAU-NF-Y610X) and 5'-TATGGATGCGCAAT ATNNKGGATATCTTTGG-3' and 5'-CCAAAGATCATCCM NNATTCGGGCCATCATA-3' for Y611X (pDAU-NF-Y611X), respectively (N, mixture of A, T, G and C; K, mixture of G and T; M, mixture of A and C).

The plasmid for the production of FLAG tagged-neurolysin in yeast was constructed using pYES2 vector (Invitrogen, CA, USA). The C-terminal of neurolysin is structurally located away from the catalytic domain, therefore, additions of a FLAG tag at the C-terminal likely would not affect the activity of neurolysin. The DNA fragment encoding neurolysin was amplified by PCR using the primers 5'-GTATGGATGCGCAACCATCACCACATCCCATGAC-ACACCTGGGAAAAAGGCGTCCCTCTCCTCTCAAGC- ATGCTCTCACTCACGTGCGTGGCAAGG-3' and 5'-TCACGT TGGGATCTACGCAACCATTCCGGCTCGACTCATTA GGAATGCTTTTTGGTTTGGCTCACGTTGCAAGAAATT TGGATCCTTACGAACCATTCAGGCCTCGACTCATTA-3'. The fragment was digested by KpnI and BamHI and introduced into the pKpnI–BamHI site of the pYES2 vector. Moreover, a FLAG tag-encoding DNA sequence was added on downstream of the neurolysin encoding DNA sequence by site-directed mutagenesis using the primers 5'-GATCATCCTAATGGATGCGCAACCATCACCACATCCCATGAC-ACACCTGGGAAAAAGGCGTCCCTCTCCTCTCAAGC- ATGCTCTCACTCACGTGCGTGGCAAGG-3' and 5'-CACACTCGCCCGCCGTATTACTTAGGTGGTCCAGG-3' and 5'-CACACTCGCCCGCCGTATTACTTAGGTGGTCCAGG-3'. The resulting plasmid was denoted pECU-HNF.

The plasmid for the production of FLAG tagged-Y610L mutant neurolysin was also constructed from pECU-HNF plasmid by site-directed mutagenesis using the primers 5'-GGGTATGATGGCCAANNKTATGGATATTGTG-3' and 5'-CCAAAGATCATCTAMTNNTTGCCATCATA-3', and denoted pECU-HNF-Y610L. The sequences of constructed plasmids were confirmed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, CA, USA). Displays of wild-type and mutant neurolysins on yeast cell surface were collected by centrifugation at 800 x g and washed with phosphate-buffered saline (PBS). The peptide-cleaving reaction was performed with the following concentrations of yeast and peptide, OD600 = 50 and 100 µM, respectively, in a total volume of 300 µl of PBS at 37°C for 30 min with gentle shaking. After the reaction, the cells were eliminated by centrifugation at 800 x g and the increase in fluorescence at λex = 330 nm and λem = 390 nm in the supernatant was measured to quantify the concentration of cleaved peptide with Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) using a tissue-culture plate (353072 Multiwell 96-well; Becton–Dickinson Labware, NJ, USA). At least two independent experiments were performed for each peptide.

Screening of mutant neurolysins

Individually six comprehensive libraries of neurolysin (D467X, R470X, E510X, Y606X, Y610X and Y611X libraries, which contained 19 species of mutant neurolysins, respectively) were examined for the screening. Fifty colonies were selected from each library and measured for catalytic activity of displayed mutant neurolysin on the yeast cell surface towards the MMPs-2/9- and -3-specific peptides. Substrate specificity of mutant neurolysin was estimated by activity ratio calculated by following formula: (Activity ratio) = (RFU value of the MMP-3-specific peptide)/(RFU value of the MMPs-2/9-specific peptide). The colonies, which showed an activity ratio < 1, were cultivated and their plasmids were sequenced.

Purification of tagged-neurolysin

Yeast cells transformed by pECU-HNF or pECU-HNF-Y610L were cultivated in SGC+W medium at 30°C. These transformants produced and stored the FLAG-tagged wild-type or Y610L mutant neurolysin within their cell. Collected cells were suspended in PBS containing 1 µg/ml of pepstatin A, and subjected to five cycles of freeze–thawing. After centrifugation, the supernatant was mixed with anti-FLAG M2 affinity gel (Sigma, MO, USA) equilibrated with PBS at 4°C. The resin was washed and suspended with PBS for removal of non-specific proteins, and FLAG tagged-neurolysin was eluted from the resin using a 3× FLAG peptide (Sigma). Homogeneity of purified neurolysin was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Steady-state kinetics of hydrolytic activity

The hydrolytic reaction for the MMPs-2/9 or the MMPs-3-specific fluorescent-quenching peptides were determined in PBS, containing 5 nM wild-type or mutant neurolysins and 1.25–50 µM substrates at 37°C by recording an increase in fluorescence at λex = 330 nm and λem = 390 nm. The parameters Kcat and Km were evaluated by Hanes–Woelf plot (Hanes, 1932). The average values of three independent experiments were shown with standard errors in Table I.

Results

Selection of mutation residues

A structural comparison between neurolysin and MMP-9 showed that the catalytic domain of neurolysin is very similar to that of MMP-9 (Kadonosono et al., 2007a). On the substrate recognition surface of neurolysin, the tyrosine-rich loop (604-GGGDQYYGY-613) (Fig. 2A) may be involved...
in the recognition of the substrate P2' amino acid as a counter element of the substrate P2' amino acid recognizing the wall-forming segment of MMP-9 (Kadonosono et al., 2007a). This loop can move near the catalytic center for binding to the substrate (Brown et al., 2001; Comellas-Bigler et al., 2005), and the Tyr606 and Gly608 residues of the loop are involved in the formation of the neurolysin S1 subsite (Machado et al., 2007). In the crystallographic structure of a closely related dipeptidyl carboxypeptidase (Dcp) in the presence of an octapeptide inhibitor (Comellas-Bigler et al., 2005), the Tyr611 residue, which corresponds to the Tyr610 residue of neurolysin, contributed to the fixing of the C-terminal carboxylate group of the P2' amino acids of substrates. These findings suggest the importance of tyrosine residues in the loop for substrate recognition. Therefore, Tyr610 and its surrounding tyrosine residues, Tyr606 and Tyr611 (Fig. 2B), were comprehensively substituted.

Arg470 locates on the channel wall opposite the 604–613 loop (Fig. 2A), presumably forming the neurolysin S2' subsite in computational model (Ray et al., 2002). When Arg470 and Thr499 were substituted to Glu and Arg, respectively, the substrate specificity of mutant neurolysin was markedly altered compared with that of wild-type neurolysin (Lim et al., 2007), suggesting that Arg470 and its surrounding residues contributed to the recognition of substrates. Therefore, three residues, Asp467, Arg470 and Glu510 (Fig. 2B), were also comprehensively substituted.

**Evaluation of mutant neurolysins from library**

After screening using the MMPs-2/9- or the MMP-3-specific fluorescence-quenching peptides, seven mutant neurolysins showed a strong preference for the MMP-3-specific substrate (Fig. 3). The amino acid differences between the two peptides existed in three positions, tyrosine or valine at P1', alanine or glutamate at P2' and methionine or arginine at P5', however, by referring to substrate recognition motifs of neurolysin, the alanine/glutamic acid change in the P2' position was fatal for the cleavage. Although six residues were individually and comprehensively substituted, substitutions at only two positions, nos. 470 and 610 amino acid residues, were obtained, indicating that both residues are involved in the recognition of substrate P2' amino acid. No clones from

---

**Table 1. Steady-state kinetic parameters of wild-type and Y610L mutant neurolysins**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ ($\mu$M)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$μM$^{-1}$)</th>
<th>Activity ratio$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>MMP-2/9</td>
<td>2.1 ± 0.37</td>
<td>7.7 ± 2.2</td>
<td>0.29 ± 0.028</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>MMP-3</td>
<td>1.9 ± 0.083</td>
<td>19 ± 4.1</td>
<td>0.10 ± 0.016</td>
<td></td>
</tr>
<tr>
<td>Y610L mutant</td>
<td>MMP-2/9</td>
<td>0.76 ± 0.057</td>
<td>14 ± 2.7</td>
<td>0.057 ± 0.0070</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>MMP-3</td>
<td>1.1 ± 0.05</td>
<td>6.0 ± 0.48</td>
<td>0.19 ± 0.015</td>
<td></td>
</tr>
</tbody>
</table>

$^a$(Activity ratio) = ($k_{cat}/K_m$ value of the MMP-3 substrate)/($k_{cat}/K_m$ value of the MMP-2/9 substrate)
nos. 467, 510, 606 and 611 libraries exhibited a change in substrate specificity. Not all of the possible substitutions might have been screened because only 50 colonies were selected from each library. Into the position of no. 470 residue, a basic or a polar amino acid was introduced. The R470K and R470H mutants had basic residues that were the same as those of wild-type neurolysin; therefore, we expected them to show specificities similar to that of wild-type neurolysin. However, the specificities of the mutants were altered, suggesting that the side chain of the introduced lysine or histidine is smaller than that of arginine which affected the recognition of substrate P2′ amino acid. Polar amino acids, namely, threonine and serine, were also introduced into the 470 residue position. The hydroxyl group of these amino acids may contribute to the recognition of substrate P2′ amino acid, presumably by hydrogen bonding. The R470S mutant showed a stronger preference for the MMP-3-specific substrate than the R470T mutant. This also suggests the importance of a small side chain for the preference towards the MMP-3-specific peptide. On the other hand, hydrophobic leucine, isoleucine and valine were introduced into the 610 residue position. By this substitution, the hydrophobicity of the neurolysin S2′ subsite increased together with the decrease in side chain size. However, the substrate specificity of the Y610L mutant was different from that of the Y610L mutant, although isoleucine and leucine shared the same properties, indicating that the shape of the side chain is also involved in the recognition of substrate P2′ amino acid.

To evaluate the effect of the simultaneous substitution at Arg470 and Tyr610 on substrate specificity, four plasmids for displaying double mutant neurolysin, R470T/Y610L, R470T/Y610V, R470S/Y610L and R470S/Y610V mutants, were generated by site-directed mutagenesis. The substrate specificities of these mutants were determined by a previously described method; however, no mutant showed a stronger preference for the MMP-3-specific substrate than the Y610L single mutant (data not shown). Thus, it was determined that the Y610L mutant neurolysin had the strongest preference toward the MMP-3-specific substrate in this research.

**Steady-state kinetics of Y610L mutant neurolysin**

The steady-state kinetic parameters of wild-type and Y610L mutant neurolysin were measured to determine the difference in substrate specificity using purified enzymes produced on the basis of screened clones as described in the experimental procedure (Table I). The $k_{cat}$ values of wild-type neurolysin toward the MMPs-2/9- or -3-specific peptides were almost the same; however, $K_m$ toward the MMPs-2/9-specific peptide was about threefold lower than that toward the MMP-3-specific peptide, indicating that the strong binding ability toward the MMPs-2/9-specific peptide resulted in an effective hydrolysis of the peptide, as shown by $k_{cat}/K_m$ values. On the other hand, the $k_{cat}$ values of the Y610L mutant neurolysin toward these two peptides were also similar; however, the $K_m$ toward the MMPs-2/9-specific peptide was about 3-fold higher than that toward the MMP-3-specific peptide. This preference for the MMP-3-specific peptide resulted in catalytic activity toward this peptide that is higher than that toward the MMPs-2/9-specific peptide, as shown by the $k_{cat}/K_m$ values. Moreover, the $k_{cat}$ values of the mutant were lower than those of the wild-type enzyme, indicating that the substitution of Tyr610 decreased the catalytic efficiency.

**Discussion**

Although a number of neurolysin residues were comprehensively substituted to enhance its binding ability toward the MMP-3-specific peptide, which contains charged glutamic acid at the P2′ amino acid position, the substitution of Tyr610 with hydrophobic leucine was most effective, suggesting that P2′ amino acids of substrates bind to the neurolysin S2′ subsite by hydrophobic interaction, and not by charge interaction of both side chains. Among neurolysin and closely related peptidases, the crystallographic structure of Dcp has already been determined with a peptidic inhibitor (protein data bank (PDB) code: 1Y79). The complex structure suggests that these peptidases, including neurolysin, exhibit two conformations during hydrolysis: the open form without substrates and the closed form with substrates (Comellas-Bigler et al., 2005). As the known structure of neurolysin (PDB code: 1I1I) is the open form, the closed form of neurolysin would be evaluated using the complex structure of Dcp. A structural comparison between neurolysin and closely related Dcp revealed that the substrate-binding wall of neurolysin closes and the putative neurolysin S2′ subsite comprising Tyr610 and Tyr613 also moves near the side chain of the P2′ amino acid of the substrate during binding. In wild-type neurolysin, the two benzene rings of Tyr610 and Tyr613 form a closed neurolysin S2′ subsite with preferences for hydrophobic and aromatic amino acids; therefore, the hydrophobic alanine residue in the MMPs-2/9-specific peptide was preferred (Fig. 4A). On the other hand, the Leu610 residue in the Y610L mutant neurolysin forms a hydrophobic neurolysin S2′ subsite that is wider than that of wild-type neurolysin because the van der Waals volume and hydrophobicity of leucine are smaller and stronger than those of tyrosine, respectively (Fig. 4B). The enlarged neurolysin S2′ subsite could bind only to carbon atoms of the side chain of glutamic acid in the MMP-3-specific peptide by hydrophobic interaction. Carboxylate charge of the glutamic acid might be neutralized by nitrogen atoms of side chains of nearby Gln555 and/or His601, or by solvent. The importance of the shape of the neurolysin S2′ subsite was supported by the finding that the preference of the Y610L mutant toward the MMP-3-specific peptide was weaker than that of the Y610L mutant. The isoleucine residue had the same van der Waals volume and hydrophobicity as the leucine residue, but the shapes of their side chains were different, resulting in a difference in the shape of the neurolysin S2′ subsite between the Y610L and Y610L mutants (Fig. 3). Moreover, the $K_m$ of the Y610L mutant neurolysin toward the MMPs-2/9-specific peptide was higher than that of the wild-type enzyme, indicating that the reformed neurolysin S2′ subsite of the mutant was not suitable for binding to the P2′ amino acid of the MMPs-2/9-specific peptide, although it was suitable for binding to the P2′ amino acid of the MMP-3-specific peptide.

The $k_{cat}$ of the Y610L mutant neurolysin was lower than that of wild-type neurolysin. One putative explanation of this change was that the reaction–transient structure composed of the enzyme and the substrate was destabilized by the
The substitution at position 610. This destabilization may be caused by the deviation of Tyr613 in the mutant. That is, the position of Tyr613 was fixed by Tyr610 by a stacking interaction, and the hydroxyl group of Tyr610 played a role in the catalytic reaction as an oxyanion hole in wild-type neurolysin (Oliveira et al., 2003). The substitution of Tyr610 with a non-aromatic leucine residue resulted in the loss of stacking interaction; therefore, Tyr613 of the Y610L mutant might be deviated with decrease in catalytic efficiency.

There are few trials on the modification of substrate specificity of peptidases. This is because much difficulty exists in the selection of amino acid species for the generation of modified subsites, even if the crystallographic structures or substrate recognition residues of peptidases have already been determined. A directed evolution approach has already been applied to alter the substrate specificity of E. coli endopeptidase OmpT using the E. coli display system (Varadarajan et al., 2005). This approach requires a high throughput screening technology and the E. coli display system has limitations in the molecular sizes of display-proteins, therefore, it is difficult to apply this method to alter the substrate specificity of peptidases in the cases where high throughput screening system is not ready or the target peptidase has a large molecular mass. Here, a rapid strategy for the modification of the substrate specificity of peptidases was developed on the basis of the semirational protein engineering method coupled with the use of the yeast molecular display platform. As a result, the residues and mechanisms of substrate binding at the neurolysin S2’ subsite were clearly revealed. Peptidases are an attractive molecular tool for eliminating toxic peptides in foods, regulating the amount of bioactive peptides for health, screening of inhibitors for drug design and synthesizing valuable peptides for supplements, among others. Therefore, studies on tailor-made modification of the substrate specificity of peptidases would be expected to open a novel field called ‘peptidase engineering’.

**Funding**

This work was partially supported by the CoE for Microbial-Process Development Pioneering Future Production Systems from the Ministry of Education, Science, Sports and Culture, Japan, and the Research and Development Program for New Bio-industry Initiatives.

**References**


Received November 23, 2007; revised April 11, 2008; accepted April 11, 2008

Edited by Alan Berry