Chemistry & Biology, Vol. 10, 53–60, January, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S1074-5521(02)00308-3

Construction of a Protein-Detection System Using a Loop Peptide Library with a Fluorescence Label

Mizuki Takahashi,[1] Kiyoshi Nokihara,[2] and Hisakazu Mihara[1,*]
[1]Department of Bioengineering
Graduate School of Bioscience and Biotechnology
Tokyo Institute of Technology
Yokohama 226-8501
[2]HiPep Laboratories
Nakatsukasacho 486-46, Kamigyo-ku
Kyoto 602-8158
Japan

Summary

Construction of a novel protein-detection system was carried out using a designed peptide library with fluorescent labels based on loop structures. As a basic model study, detection of α-amylase using fluorescent-labeled peptides derived from an active loop of tendamistat was examined. The detection methods for proteins with immobilized peptides as well as peptides in solution have been successfully established. Based on these results, a loop peptide library that has various turn sequences grafted on a stable loop structure has been constructed. Various proteins with recognition patterns corresponding, for instance, to "protein fingerprints" could be detected using an immobilized peptide library. The present results suggest that the system can be applied to the development of a peptide microarray that behaves as a protein chip.

Introduction

Recent remarkable advances in genome projects have resulted in vastly increased knowledge of the genomic sequences of humans and other organisms. Accordingly, a number of technologies have been developed to investigate cellular events on a genome-wide scale. For example, oligonucleotide arrays provide information on changes in mRNA expression levels in response to a variety of physiological stimuli [1]. The protein expression levels, however, often do not correlate with mRNA levels [2]. In most cases, a gene function is manifested by the direct activity of its translated proteins and, in addition, a variety of posttranslational modifications of these take place. Hence, providing superior approaches for elucidating gene and cell functions is indispensable for analyses of protein functions. For the elucidation of structures and/or functions of proteins, technologies such as two-dimensional gel electrophoresis [3] or other chromatographic separation methods in conjunction with mass spectrometry [4, 5] have been developed.

Recently, pioneer works directed toward the production of protein microarrays have been reported [6–11]. Protein chips or microarrays using immobilized proteins and/or antibodies are regarded as high-throughput methods for simultaneous parallel detection of target molecules [6]. In addition to the detection method, the choice of capture molecules that are immobilized onto a chip or array is definitely one of the most important aspects of protein chip construction. Hence, purified proteins and/or monoclonal antibodies together with well-designed peptides as capture molecules should have great potential, as peptides can be easily designed and prepared by well-established methods. In this respect, small peptides used for protein recognition are believed to have limited affinities and specificities. However, it is known that designed peptides that can assume appropriate structures are recognized by proteins with high affinities, for example, in MHC binding [12]. Since peptide libraries with a designed structure can be synthesized systematically, proteins can be detected and classified by their recognition patterns. An additional advantage of such peptides is that chemical synthesis allows labeling with fluorescent dyes at any position in the peptide sequence. Thus, proteins in analytes can be detected through their interaction with the labeled peptides and need not be labeled themselves.

Using these as our criteria, we have attempted to construct a protein-detection system using designed peptide libraries with suitable secondary structures. The peptides labeled with fluorescent dyes were used for the arrays, and proteins were detected through changes in fluorescence intensities. Hence, loop structures were chosen as the structural motif, as these are found on the surface of proteins and have often been implicated in the recognition of protein binding partners. An antigen binding site of antibody is composed of a combination of six loop structures [13]. Protease binding sites of protease inhibitors comprise a short extended peptide stretch with varying sequences that are present as an exposed loop in the structural framework. That is, binding loops of different inhibitors adopt a similar conformation [14]. Therefore, loop structures derived from proteins are important structural motifs as interaction sites with other molecules.

Here, we have demonstrated the techniques required for the construction of a protein-detection system using a peptide library based on a loop structure. Initially, the detection methods were established in solution and/or immobilized on the solid phase using a model system of loop peptides that were expected to interact with the protein α-amylase. Hence, a loop peptide library was constructed based on a designed structure, and responses against various proteins were examined using an immobilized peptide library. Throughout this study, the possible use of the structure-designed peptide array for the development of protein chips has been emphasized.

Results

Design and Synthesis of Model Peptides

In order to establish the assay procedure, peptides with a secondary structure were designed on the basis of

*Correspondence: hmihara@bio.titech.ac.jp

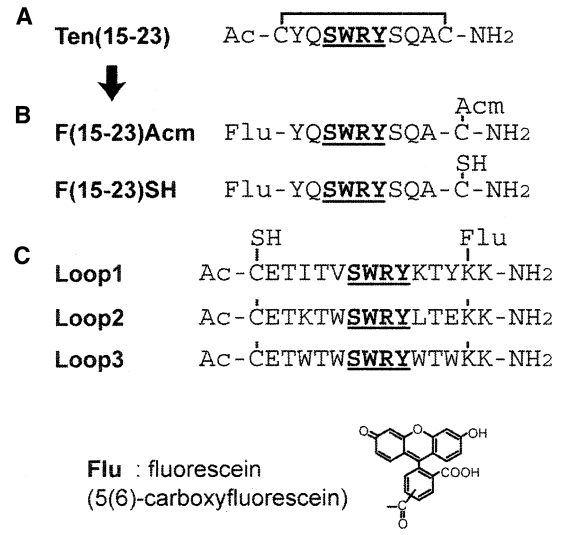

Figure 1. Design of β Hairpin Peptides with a Fluorescent Probe

(A) Sequence of a peptide Ten(15-23) derived from α-amylase binding site of tendamistat [17].

(B and C) Design of peptides with a fluorescent probe based on Ten(15-23) sequence (B) and de novo designed β hairpins; loop 1 [18, 19], loop 2 [20], and loop 3 [21] (C) for the detection of α-amylase.

a model peptide-protein system, and hence we have focused on the interaction between α-amylase and tendamistat. Tendamistat is a β sheet protein consisting of 74 amino acids derived from *Streptmyces tendae*, which has strong α-amylase inhibitory activity [15]. The three-dimensional structure of tendamistat complexed with α-amylase [16] revealed that one of the loops in tendamistat forming a type I β turn interacts with the catalytic site of α-amylase. This loop contains the conserved residues Trp^{18}-Arg^{19}-Tyr^{20}, which are necessary for binding and the inhibitory activity. Recently, peptides derived from the α-amylase binding site of tendamistat were reported as potent α-amylase inhibitors. One of these, Ten(15-23), is composed of 11 amino acid residues with an intramolecular disulfide (Figure 1A) [17]. Hence, the fluorescent-labeled sequence of Ten(15-23) was used as a scaffold (Figure 1B) with 5(6)-carboxyfluorescein (fluorescein) as a fluorescent probe attached to the N terminus of the peptide. The side chain of the C-terminal Cys residue was protected with an Acm group or intact sulfhydryl. The latter was used for selective immobilization onto a solid support described below.

Further, we have designed more stable β hairpin to acquire a better response. Three de novo-designed β hairpins were considered as scaffolds (Figure 1C) [18–21]. In the design of the position in the strand, the turn sequence of the active site of tendamistat (Ser-Trp-Arg-Tyr) was added in order to retain the binding site for α-amylase. A fluorescein moiety was introduced into the side chain of the C-terminal Lys residue. A Cys residue with a thiol function was introduced at the N terminus to immobilize the peptides onto a solid support. The fluorescent changes of these designed peptides were monitored by addition of proteins.

Structural Analyses Using Circular Dichroism Spectroscopy

In order to evaluate the structural properties of the designed peptides, the far-UV circular dichroism (CD) spectra of synthesized peptides were measured. As shown in Figure 2, F(15-23)Acm and the designed Loop2 showed an almost random conformation in solution. This suggests that the absence of a disulfide bond, in the cases of F(15-23)Acm and Loop2, affects the conformational stability, even though a salt bridge had been introduced instead of a disulfide bond in the case of Loop2 [20]. In contrast, the spectra of Loop1 and Loop3, which were designed to form stable β-hairpin structure, showed a minimum at 215 nm and a maximum at 195 nm, which suggests the presence of a definite population of structured conformations corresponding to that expected for a β sheet mixed with a β turn [18, 22]. It was impossible to obtain the binding conformation of the peptides with α-amylase because of strong CD signals derived from the protein.

Detection of α-Amylase Using Designed Peptides in Solution

Fluorescence changes by the addition of α-amylase were measured in the buffer solution in order to compare the responses to the designed peptides. In the case of the peptide with the sequence derived from tendamistat, F(15-23)Acm, fluorescence intensity changes of ~1.5 times were observed with the addition of α-amylase (Figure 3A). On the other hand, only negligible changes were observed with the addition of bovine serum albumin (BSA) as a reference protein, suggesting that the peptide has specificity for the binding of α-amylase. From the fluorescence changes, the binding constant of F(15-23)Acm with α-amylase was calculated as $1.8 \times 10^6\ M^{-1}$ (Figure 3A) using a single-site binding equation [23]. Additionally, we also attempted to detect the protein using peptides labeled with other fluorescent probes, such as 5-dimethylaminonaphthalene-1-sulfonyl (dansyl), 7-diethylaminocoumarin-3-carboxylic acid (coumarin), and *N*-(acetaminoethyl)-1-naphthylamine-5′-sulfonic acid (EDANS) instead of the fluorescein moiety. However, these peptides were much less responsive, and there were some difficulties in the fluorescence measurements due to the weak fluorescence intensities in solution and also in the solid state (data not shown). Hence, the fluorescein moiety was chosen as the optimal fluorophore, as it showed good intensity changes that allowed protein detection.

In the cases of the peptides with designed hairpin sequences, all three peptides (Loop1–3) showed remarkable increases (>4 times) in the fluorescence intensities by the addition of α-amylase, while only small changes were observed by the addition of BSA (Figures 3B–3D). These results indicated that the designed peptides could also interact with α-amylase in an effective and specific manner, and suitable structural design to form a relatively stable loop structure caused a higher response against the target protein. The binding constants of these peptides with α-amylase were also calculated from the fluorescence changes (Figures 3B–3D).

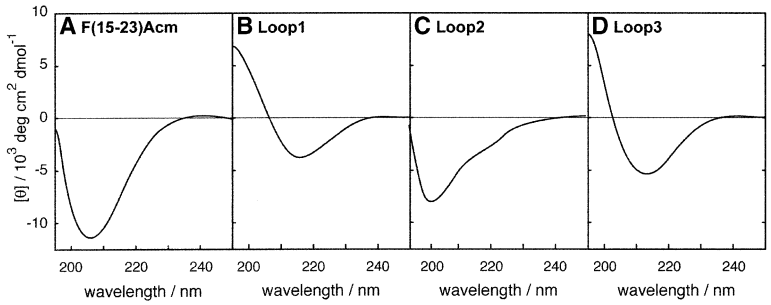

Figure 2. Circular Dichloism Spectra of Designed Peptides

(A) F(15-23)Acm (4.5 μM); (B) Loop1 (25 μM); (C) Loop2 (52 μM); (D) Loop3 (20 μM). In 20 mM Tris-HCl, 150 mM NaCl (pH 7.4) at 25°C.

As shown in Figure 3, the peptide Loop1 has a relatively strong affinity for α-amylase, although it is weaker than that of F(15-23)Acm. The binding constant of Loop3 was much weaker, suggesting that the bulky Trp residues in the Loop3 scaffold may cause inefficient interaction with the protein. In addition, the structural instability of Loop2, which was suggested from the CD studies described above, may cause a weaker binding affinity with the protein than that of Loop1.

Immobilization of the Designed Peptides for Detection of Proteins

Detection of proteins using immobilized peptides on a solid surface was also attempted in order to construct a high-throughput detection system. In this study, a 96-well microtiter plate was used as a solid support, and a 'hydrocoating' technique, which is the covalent immobilization of biomolecules in highly hydrophilic surroundings produced by the dextran coating [24], was applied to immobilize peptides onto the plate. The outline of the method for the immobilization is illustrated in Figure 4A. Initially, a polymer surface (polystyrene) of a microtiter plate was coated with poly-L-lysine to produce an amino-functionalized surface. 2,2,2-Trifluoroethanesulfonyl (tresyl)-activated dextran was attached covalently to the surface, leaving a sufficient number of active groups for secondary binding of other biomolecules which have amino or thiol groups. In this case, further modifications of the surface by diamine and bromoacetic acid were successively carried out for the selective attachment of peptides using the reaction between Cys-sulfhydryl and bromoacetyl groups. The peptides were immobilized onto the microtiter plate and used for detection of α-amylase and other proteins.

Figure 4B shows the fluorescence change of F(15-23)SH and the three designed peptides immobilized onto the plate by the addition of various proteins. The protein solutions were added to each well containing the immobilized peptide, incubated for 24 hr at 4°C, and then the fluorescence intensities (I) were measured by a microplate reader. After removal of solutions and washing with water, only buffer was added to the wells, and the fluorescence intensities of immobilized peptides were measured relative to their initial intensities (I_0). The responses to the addition of the proteins were evaluated using the value calculated as an increment of the initial intensity without protein (I/I_0).

As shown in Figure 4B, Loop1 gave the highest response with the addition of α-amylase, while Loop3 showed only negligible changes. Loop1 showed a higher response toward α-amylase than F(15-23), suggesting that this is due to the relatively stable loop structure achieved by the appropriate structural design. In the cases of Loop2 and Loop3, however, the responses upon addition of the protein became smaller on immobilization. It is perceived that there is some difficulty in forming suitable conformations in the immobilized state. In addition, Loop1 showed the fluorescent responses against α-amylase in a dose-dependent manner, suggesting that the fluorescence responses were relevant to the binding affinity (Figure 4C). The protein α-amylase can be detected at a lower concentration of 5 μg/ml.

Furthermore, five proteins from the glycosidase family, including α-amylase, β-glucosidase, β-galactosidase, lysozyme, cellulase, and two other proteins (BSA and

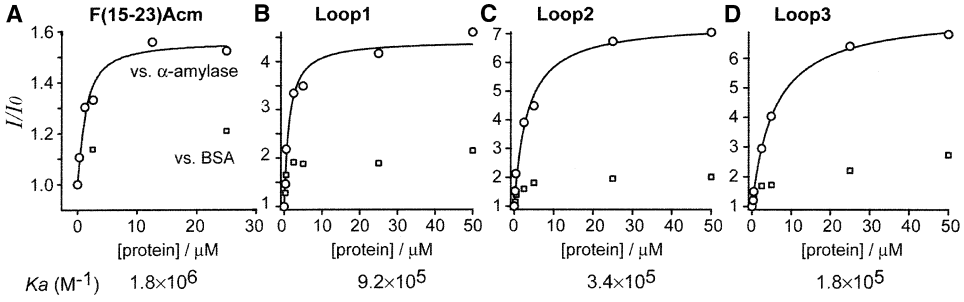

Figure 3. Fluorescence Changes of the Peptides upon Addition of α-Amylase or BSA in Solution

(A) F(15-23)Acm (1.0 μM); (B) Loop1 (0.32 μM); (C) Loop2 (0.66 μM); (D) Loop3 (0.39 μM). λex = 485 nm, λem = 530 nm in 20 mM Tris-HCl, 150 mM NaCl (pH 7.4) at 30°C.

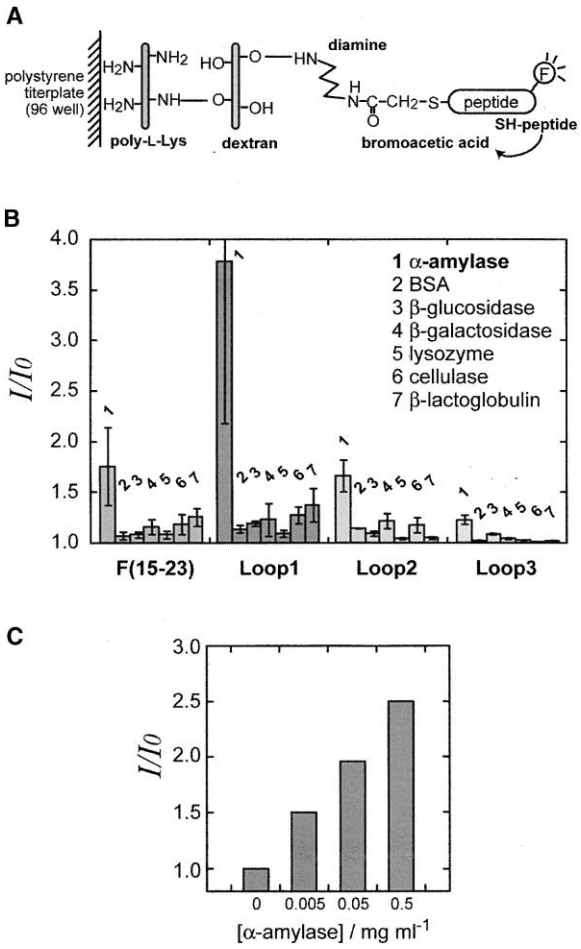

Figure 4. Fluorescence Responses of Immobilized Peptides upon Addition of Proteins

(A) Schematic representation of the immobilization of peptides onto microtiter plate [24].

(B) Fluorescence change of immobilized peptides upon addition of proteins (0.5 mg/ml).

(C) Dose-dependent detection of α-amylase by the immobilized Loop1 peptide. λex = 485 nm, λem = 530 nm in 20 mM Tris-HCl, 150 mM NaCl (pH 7.4) at 30°C.

β-lactoglobulin) were added to the immobilized peptides, and the fluorescence changes were observed (Figure 4B). The results indicated that all the peptides responded specifically to α-amylase, although the responses upon the addition of some proteins were, in some cases, very small. From these results, it was elucidated that the designed sequences were specific for the binding of α-amylase even on the solid support. Thus, the present strategy using a well-designed loop structure can be effectively applied to design peptide microarrays for detection of various proteins.

Design of a Loop Peptide Library for Detection of Various Proteins

According to the above results, a loop peptide library was constructed to detect not only α-amylase but also various proteins in order to develop microarrays with synthetic peptides. One of the designed peptides, Loop1, which showed a higher response to the target protein and some structural suitability, was used as a scaffold for a loop peptide library. The strategy for the construction of a loop peptide library is shown in Figure 5. On the basis of the Loop1 structure, the sequence of both the C and N termini were permutated to avoid continuity of β-branched amino acids such as Val, Ile, and Thr that may cause difficulties in syntheses. Four residues at the turn position corresponding to the recognition site for various proteins were designed by the random combinations of nine amino acids (Glu, Gly, His, Leu, Pro, Arg, Ser, Tyr, and Trp) which were selected as typical features of side chain functional groups. Based on this strategy, ${}_9C_4 = 126$ sequences could be generated mathematically. These 126 sequences were randomly generated using a script described by Perl and were sorted for synthetic convenience (Figure 5).

Preparation of the Library Peptides Immobilized onto the Microtiter Plates

The designed peptide library was constructed in a parallel solid-phase synthesis using a 96-well synthetic apparatus [25]. Two sets of 63 library peptides and the peptide with the native sequence (SWRY) were synthesized at once. After assembly of the peptide backbone and removal of the 4-methyltrytyl (Mtt) group of the Lys residue, the fluorescein moiety was introduced into the side chain of Lys. After cleavage, the peptides were quickly purified by gel permeation chromatography.

The resulting peptides were immobilized onto microtiter plates in the same manner as described above. Two plates were prepared for a series of a loop peptide library composed of 126 peptides. In addition, several plates were separately prepared for the same peptides for use in a simultaneous assay and to check uniformity between several plates. The correlation between fluorescence intensities of immobilized peptides on the different plates was relatively good (Pearson's correlation coefficients (P) = 0.942). This implies that the peptide immobilization process was well reproducible. In addition, the correlation between repeated experiments of protein binding with the same plate was also good (P = 0.999). Thus, this clearly indicates the fluorescein-labeled peptides immobilized onto the microtiter plates could be used repeatedly, at least several times.

Detection of α-Amylase Using the Loop Peptide Library

The fluorescence changes (I/I_0) of these immobilized peptides were now measured by the addition of α-amylase (Figure 6). As shown in Figure 6, some peptides such as 47 (LWPH), 62 (PRWL), 83 (WHPR), and 113 (PYRW) show higher responses against α-amylase, while others were much weaker. Generally, the peptides that show relatively higher responses against α-amylase involve Trp, Arg, and Ser residues in the turn region, which are conserved residues in the α-amylase binding loop of tendamistat. This result suggests that the detection pattern of protein by the loop peptide library with various turn sequences could be applicable to characterization of proteins.

According to these results, the fluorescence changes

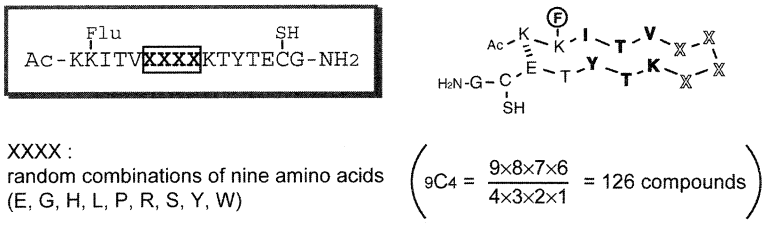

Figure 5. Design of a Loop Peptide Library

Basic structure of the library peptide based on the Loop1 structure and sequences at loop position of the synthesized library. The sequences were generated as random combinations of nine amino acids using a script described by Perl and were sorted for synthetic convenience.

LHGE	1	SHEG	21	RGEH	41	PEWL	61	YHLR	81	GRYS	101	SPHY	121
LPGE		PREG		LYEH		PRWL		SGPR		HWYS		SRHY	
RPHE		WSEG		WRLH		GHYL		WHPR		GLEW		WSLY	
YGLE		SPHG		GWLH		RWYL		WSPR		YHGW		PWLY	
WRLE		WSHG		GRPH		YWGP		WESR		RLGW		WERY	
WYLE		YSLG		RSPH		YLHP		HWSR		SLGW		RSWY	126
HGPE		WHPG		LWPH		LRHP		SPYR		LEHW			
LHPE		YHPG		LSRH		HGLP		GWYR		YRHW			
HSPE		WRPG		LWSH		LERP		GRES		PSHW			
YSPE	10	LSPG	30	LYSH	50	GLRP	70	PWGS	90	EYHW	110		
YWPE		RYPG		GEWH		LYRP		PYGS		YGLW			
LYPE		LERG		SGYH		GESP		RGHS		EHRW			
LGSE		YHRG		RYEL		ELSP		WPLS		PYRW			
WHSE		RHWG		WPGL		ERSP		ERLS		LESW			
YHSE		SYWG		SGHL		LRSP		EYLS		EYSW			
PWSE		WEYG		HSPL		GLYP		EHRS		HPYW			
PHWE		EHYG		EHRL		SWYP		GLRS		RHEY			
GPWE		ERYG		HESL		PWER		GWRS		HPEY			
PGYE		LRYG		YPSL		WEGR		REYS		WLHY			
RPYE	20	ESYG	40	RWSL	60	HLGR	80	RLYS	100	RPHY	120		

of some representative peptides by the addition of α-amylase were measured using isolated peptides in the buffer solution. As expected, three peptides, 113 (PYRW), 83 (WHRP), and 62 (PRWL), that showed a strong response against α-amylase in the immobilized manner also afforded remarkable changes in the fluorescence intensities by the addition of α-amylase in solution (2.5–3.0 times). On the other hand, the peptide 72 (GESP) showed only a small change in the solution as well as immobilized in the solid phase. From the fluorescence changes of the peptides in solution, the binding constants of these peptides with α-amylase were calculated. The peptides 113 (PYRW), 83 (WHPR), and 62 (PRWL), which showed strong responses to α-amylase in the solid-phase assay, also gave strong affinities in solution (*K*a; approximately 4×10^6, 2×10^6, 5×10^5 M^{-1}, respectively), while the peptide 72 (GESP) afforded a much smaller value (1×10^5 M^{-1}). These results strongly suggested that the responses by the addition of the protein to the immobilized peptides were almost parallel to the binding affinities of the peptides to the protein in solution.

Detection of Various Proteins Using an Immobilized Peptide Library

The fluorescence changes of peptides immobilized onto microplates by the addition of various proteins were further examined. Five proteins from the glycosidase family (α-amylase, β-glucosidase, β-galactosidase, lysozyme, and cellulase), two other proteins (BSA and β-lactoglobulin), and their mixtures (0.25 mg/ml each) were used as model proteins. An almond meal was used as a model of a practical protein mixture, as it contains β-glucosidase, β-galactosidase, and α-mannosidase activities. Trypsin treated with phenylmethylsulfonyl fluoride (PMSF) was also used, because it is known that the trypsin inhibitors such as BPTI have a conserved loop structure as a binding site for the enzyme [14].

The response of each peptide to various proteins was visualized as a black-red-yellow color from weak to strong, respectively (Figure 7). This picture indicated that peptides have specific responses to a certain protein and/or a strong response to various proteins nonspecifically, while some peptides showed almost no response. For example, the peptide 29 (WRPG) showed

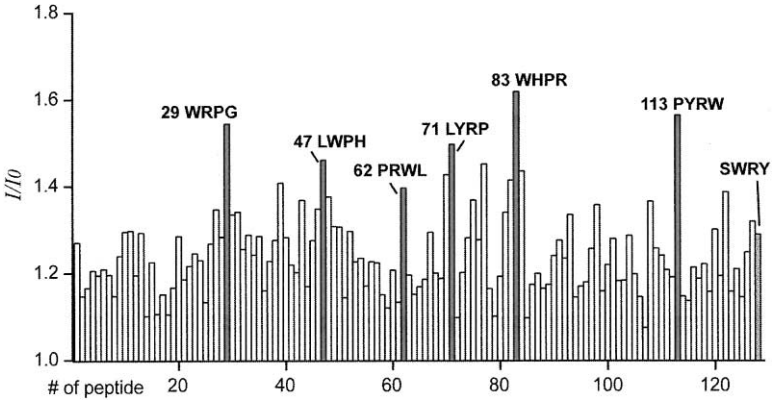

Figure 6. Fluorescence Changes of the Immobilized Loop Peptide Library upon Addition of 0.5 mg/ml α-Amylase

λex = 485 nm, λem = 530 nm in 20 mM Tris-HCl, 150 mM NaCl (pH 7.4) at 30°C.

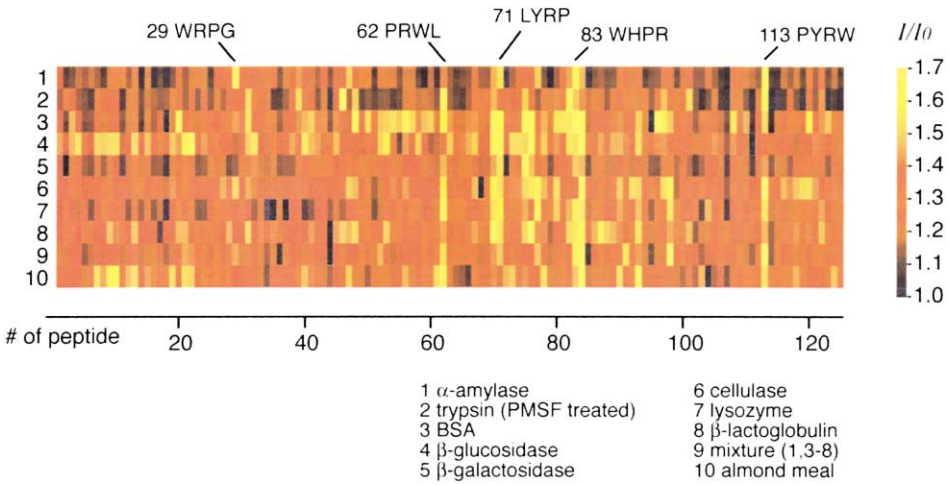

Figure 7. Protein Fingerprint Patterns Generated with the Fluorescent Responses of the Library Peptides Immobilized onto Microtiter Plates

The patterns were generated upon addition of various proteins (0.5 mg/ml except for mixture, 9; 0.25 mg/ml each). Signals were expressed using the program Igor Pro (WaveMetrics, Inc.); the fluorescent responses (I/I_0) were shown as a black-red-yellow color from weak to strong, respectively. Responses (I/I_0) are indicated as a normalized scale for each protein. λex = 485 nm, λem = 530 nm in 20 mM Tris-HCl, 150 mM NaCl (pH 7.4) at 30°C.

a strong and specific response against α-amylase but much weaker against that of other proteins. The peptide 83 (WHPR), which was suggested to be a relatively strong binder for α-amylase (Figure 6), also responded specifically to PMSF-treated trypsin. In contrast, the peptides 62 (PRWL) and 113 (PYRW), which showed a strong response against α-amylase as mentioned above (Figure 6), showed relatively strong responses against other proteins. The peptide 71 (LYRP) also responded to various proteins nonspecifically. The peptides with positive charge generally showed higher responses against α-amylase and/or trypsin, while the negatively charged peptides responded weakly. These results do not contradict data on the binding property of these proteins; the α-amylase or trypsin binding site of their inhibitors contains positively charged residue(s).

Consequently, each protein showed a characteristic pattern to each peptide in a library which could be used as a 'protein fingerprint' of that particular protein. Thus, proteins can be identified by such a peptide library. Furthermore, it was also revealed that a particular peptide gives a specific response to a definite protein in the mixture of proteins (e.g., 62, 84, 85, 113). Thus, by implication the peptide library has a great potential for the analysis of protein mixtures.

Discussion

In the present study, we have successfully constructed a protein-detection system using synthetic structurally designed, fluorescent-labeled peptides. Initially, the fluorescent probe for the effective detection of the protein was selected, and the detection methods were established in solution and also in an immobilized manner, using loop peptides derived from tendamistat and α-amylase. To obtain a higher response, the peptide loop structure was further designed to form a more stable structure, and it was revealed that the peptides with the more stable structures showed higher responses. The utilization of the peptides with an appropriate structural design and not simply a specific arrangement of amino acids possibly gives higher and more specific responses than the use of simple physically and/or chemically modified surfaces. Moreover, the fluorescence responses were dose dependent so that protein α-amylase can be detected at 5 μg/ml.

Furthermore, a loop peptide library was constructed and it was shown that the various proteins could be detected using an immobilized peptide library. A small set of peptides designed as a systematically constructed library could be used as capture molecules for protein detection systems, which can be considered as 'indicators' for the protein properties. In this study, each peptide and/or protein showed a characteristic response to proteins and/or peptides, and in some cases peptides showed a protein-specific response. Thus, various proteins were detectable with a response pattern among library peptides that were characteristic and could be regarded as a 'protein fingerprint.' In particular, the loop peptide library was suitable for detecting certain proteins such as α-amylase and trypsin which were inhibited by the protein with a conserved loop structure; that is, they can effectively bind peptides or proteins with a loop structure.

Throughout this study, we have developed a novel application of the well-designed peptide library to capture molecules in protein-detection systems such as microarrays. Further improvement on sensitivity of detection and the number of peptides together with miniaturization of the array and/or robotic handling will provide a practical protein chip.

Significance

As an improvement on genome-wide sciences, the development of protein chips/microarrays has been

highly significant for the technology, providing a high-throughput parallel detection method of target proteins. One of the most important points for protein chips is definitely the choice of capturing agents that are immobilized onto the chip. In addition to purified proteins and/or monoclonal antibodies [6–11], the well-designed peptide libraries described here also have great potential as capturing agents. In this study, we have accomplished the construction of small peptide arrays with peptides that have a loop structure. Chemical syntheses of peptides provide materials that can be readily modified with fluorescent labels introduced into any position that allows detection of proteins through changes in fluorescence intensities. Hence, the structural design of the peptides was also important. Furthermore, the synthetic peptide library with a designed loop structure was constructed systematically, and it was revealed that various proteins could be detected and classified based on their recognition patterns as 'protein fingerprints'. Throughout this study, it is strongly suggested that the peptide array described here, one of a number of novel and useful approaches to the production of protein chips, can be used for protein analyses.

Experimental Procedures

General Remarks

All chemicals and solvents were of reagent or HPLC grade and were used without further purification. Proteins were purchased from Sigma-Aldrich Japan. HPLC was performed on a system composed of a Hitachi D7500 Chromato-Integrator, a L7400 UV-VIS Detector using a Wakosil 5C18 or a YMC-Pack ODS-A (4.6 × 150 mm) for analysis or a YMC ODS A323 (10 × 250 mm) for purification, or on a Shimadzu LC2010C system using a Cadenza CD-C18 column (4.6 × 50 mm; Imtakt) with a linear gradient of acetonitrile/0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 or 3.0 ml min^{-1} for analysis or purification, respectively. MALDI-TOFMS was performed on a Shimadzu KOMPACT MALDI III mass spectrometer with 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. Amino acid analysis was carried out using a Wakopak WS-PTC column (4.0 × 200 mm; Wako Pure Chemical Industries) after hydrolysis in 6 M HCl at 110°C for 24 hr in a sealed tube and labeling by phenyl isothiocyanate (PITC).

Synthesis of Individual Peptides

Peptides were synthesized by the solid-phase method by means of Fmoc chemistry [26] on Rink amide resin using an Advanced ChemTech Model 348 MPS peptide synthesizer with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as a coupling reagent. Side chain protections were as follows: acetamidomethyl (Acm) or triphenylmethyl (Trt) for Cys; *t*-butyl (tBu) for Asp, Glu, Ser, Thr, and Tyr; Trt for His, Asn, and Gln; *t*-butyloxycarbonyl (Boc) or 4-methyltrityl (Mtt) for Lys; and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. In the case of F(15-23)Acm and F(15-23)SH, 5(6)-carboxyfluorescein (5(6)-FAM; Molecular Probes, Inc.) was coupled to the N terminus of the peptides using 5(6)-FAM (1.5 eq.), HBTU (1.5 eq.), 1-hydroxybenzotriazole (HOBt) (1.5 eq.), and diisopropylethylamine (DIEA) (3 eq.) after assembly of all amino acids. In the case of the peptides Loop1, Loop2, and Loop3, after assembly of all amino acids an Mtt group was removed by the treatment with the solution of dichloromethane (DCM)/triisopropylsilane (TIS)/TFA = 94/5/1, and the fluorescein moiety was introduced to the ε-amino group of the Lys residue by using succinimidyl ester of 5(6)-FAM (3 eq.) and DIEA (1.5 eq.) for 6–12 hr. After introduction of the fluorescein moiety, the resin and all the protecting groups except Acm were removed by 1 hr treatment of TFA/*m*-cresol/ethanedithiol/thioanisole (40/1/3/3, v/v) at room temperature. The peptides were purified by RP-HPLC and characterized by MALDI-TOFMS: F(15-23)Acm *m/z* 1721.4 ($[M+H]^+$ calcd. 1721.8); F(15-23)SH 1649.4 (1649.7); Loop1, 2306.9 (2306.6); Loop2, 2359.8 (2359.6); Loop3, 2547.6 (2547.8).

Synthesis of a Peptide Library

The designed peptide library (126 peptides; Figure 5) was synthesized on Rink amide resin using a synthetic recovery module (SRM) (HiPep Laboratories). Sixty-three library peptides and the peptide with native loop sequence (SWRY) were synthesized at once. Seven common residues at the C terminus were coupled in bulk, then the resin was dispensed to the reaction tube of a SRM, and following residues were coupled on SRM using O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 5 eq.), Fmoc-amino acid (5 eq.), and DIEA (7 eq.). After assembly of all peptides, the Mtt group was removed by treatment with DCM/TIS/TFA = 94/5/1, and the fluorescein moiety was introduced by using succinimidyl ester of 5(6)-FAM (4 eq.) and DIEA (4 eq.).

For cleavage of peptides from resin and side chain deprotection, the solution of TFA/*m*-cresol/ethanedithiol/thioanisole (40/1/3/3, v/v) was used as a cleavage cocktail. The cleavage cocktail was added to the resins in reaction tubes dropwise over 30 min, after which they were allowed to stand for 1 hr. The peptides were precipitated by the addition of diethyl ether and collected with a centrifuge. The crude peptides were quickly purified by gel permeation chromatography using Sephadex G-10 (Amersham Biosciences KK) swelled with 10% (v/v) acetic acid aqueous solution in the SRM reaction tubes. The peptides were dissolved in 200 μl of methanol, tightly capped, and stored at 4°C.

In order to estimate the concentrations of the stock solutions of the library peptides, absorbance at 490 nm of the diluted solutions of peptides was measured on a Benchmark Multiplate Reader (Bio-Rad Laboratories) with a 490 nm filter using microtiter plates (Assay Plate, IWAKI) in 20 mM Tris-HCl containing 150 mM NaCl (pH 7.4). The concentrations of stock solutions were estimated by the comparison of peptide absorbance to that of a standard peptide, Loop1, whose concentration was determined by an amino acid analysis (ε = 13200).

Spectroscopic Measurements

All the measurements were performed in 20 mM Tris-HCl containing 150 mM NaCl (pH 7.4). Fluorescence spectra were recorded on a Hitachi F-2500 fluorescence spectrophotometer with a thermoregulator using a quartz cell with 10 mm pathlength at 25°C. Fluorescence intensities of the fluorescein-labeled peptides in microtiter plates were measured on an Applied Biosystems CytoFluor 4000TR Fluorescence multiwell plate reader at 30°C. Excitation and emission filters used were 485/20 and 530/10, respectively. Circular dichroism spectroscopy was performed on a Jasco J-720WI spectropolarimeter with thermoregulator using a quartz cell with 1 mm pathlength at 25°C.

Peptide Immobilization onto 96-Well Polystyrene Plates

The 'hydrocoating' technique used here is essentially identical to that previously described in [24]. Tresyl activated dextran (TAD) was prepared from dextran (MW 74,000) with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride). TAD-modified plates were prepared as described in [24]. Briefly, a solution of poly-L-lysine (MW 70,000–150,000, 0.01 mg/ml) in carbonate buffer (0.1 M, pH 9.6) was added to polystyrene microtiter plates (Assay Plate, IWAKI, 150 μl per well), and the plates were incubated for 2 hr at room temperature or overnight at 4°C. The plates were washed and TAD (0.5 mg/ml) in phosphate buffer (10 mM phosphate, 150 mM NaCl, pH 7.2) was added to the plates (150 μl/well) and incubated for 2 hr at 4°C.

To the TAD-modified plates, 1,4-diaminobutane or diethylene glycol bis(3-aminopropyl) ether (10 mM in the carbonate buffer) was added (100 μl/well) and incubated for 2 hr at room temperature. After the washing and blocking with 0.1 M 2-aminoethanol, bromoacetic acid (BrAcOH) was introduced to the amino-functionalized plates using its anhydride, which was prepared in situ by mixing of BrAcOH and DIC in *N*-methylpyrrolidone (NMP) for 1 hr. Then, the fluorescent-labeled peptides (10 μM in 100 mM Tris HCl, pH 8.0) were added to the bromoacetic acid-modified plate (100 μl/well) and incubated for 8–10 hr at room temperature. After washing with

the phosphate buffer containing 1% Triton X-100 and dried with N_2, the plate was stored at 4°C or used for the protein binding assay as described below.

Protein Detection Assay by Using Immobilized Peptides

All measurements were performed in 20 mM Tris-HCl containing 150 mM NaCl (pH 7.4). The solutions of proteins were prepared as 0.5 mg/ml by diluting solutions of 10 mg/ml. The solutions were added to the peptide-bound plates (100 μl/well) and incubated for 24 hr at 4°C. After 24 hr, the plates were brought to room temperature, and the fluorescence intensities (I) were measured by using a microplate reader (CytoFluor) at 30°C. After washing with water, the buffer was added to the plates (100 μl/well), and fluorescence intensities (I_0) were measured again. The responses of peptides to the proteins were calculated as I/I_0.

Acknowledgments

We thank Dr. V. Wray, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany, for linguistic assistance with the manuscript. This study was in part supported by a grant of the Millennium Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

Received: September 9, 2002
Revised: November 26, 2002
Accepted: December 2, 2002

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