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Preparation of Fluorescence Quenched Libraries containing Interchain Disulphide Bonds for Studies of Protein Disulphide Isomerases

JANE C. SPETZLER[1], VIBEKE WESTPHAL[2], JAKOB R. WINTHER[2] and MORTEN MELDAL[1]

[1]Department of Chemistry and [2]Department of Yeast Genetics, Carlsberg Laboratory, Valby, Denmark

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Abstract: Protein disulphide isomerase is an enzyme that catalyses disulphide redox reactions in proteins. In this paper, fluorogenic and interchain disulphide bond containing peptide libraries and suitable substrates, useful in the study of protein disulphide isomerase, are described. In order to establish the chemistry required for the generation of a split-synthesis library, two substrates containing an interchain disulphide bond, a fluoroescent probe and a quencher were synthesized. The library consists of a Cys residue flanked by randomized amino acid residues at both sides and the fluoroescent Abz group at the amino terminal. All the 20 natural amino acids except Cys were employed. The library was linked to PEGA-beads via methionine so that the peptides could be selectively removed from the resin by cleavage with CNBr. A disulphide bridge was formed between the bead-linked library and a peptide containing the quenching chromophore (Tyr(NO_2)) and Cys(*p*Npys) activated for reaction with a second thiol. The formation and cleavage of the interchain disulphide bonds in the library were monitored under a fluoroescence microscope. Substrates to investigate the properties of protein disulphide isomerase in solution were also synthesized. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Disulphide bond formation; fluorescence quenched library; peptide library; protein

INTRODUCTION

Since their introduction by Furka *et al.* [1], Lam *et al.* [2] and Houghten *et al.* [3], peptide libraries have been used as a valuable tool in biochemistry and in the discovery of new drugs. The present work describes the use of portion mixing libraries to define specific disulphide bonded peptide substrates for protein disulphide isomerase (PDI) [4] from various species. The peptide library contained a defined internal disulphide bridge available for disulphide reduction reactions and built-in monitoring system employing Abz and Tyr(NO_2) as a fluorogenic donor–acceptor pair for direct observation of the enzymatic activity. The fluoroescent 2-amino-benzoic acid (Abz) and the quenching chromophore Tyr(NO_2) are presently the most efficient energy transfer probes described [5,6]. These were employed since the disulphide bond formation and cleavage could conveniently be monitored both in solution and on solid phase. The two chromophores were incorporated in two separate peptide chains which were connected through an interchain disulphide bond.

PDI is important for the correct folding of many secretory proteins in eckaryotes and catalyses di-

Abbreviations: Abz, 2-aminobenzoyl; DCM, dichloromethane; Dhbt, 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl; DTNP, 2,2′-dithiobis(5-nitropyridine); DTT, dithiothreitol; MALDI-MS, matrix-assisted laser desorption mass spectroscopy; NEM, 4-ethyl-morpholine; Npys, 3-nitro-2-pyridine-sulphenyl; PDI, protein disulphide isomerase; PEGA, polyethylene glycol poly-acrylamide copolymer; Pfp, pentafluorophenyl; *p*Npys, 5-nitro-2-pyridinesulphenyl; TIS, triisopropylsilane; Tyr(NO_2), 3-nitrotyrosine.

Address for correspondence: Dr Morten Meldal, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark.

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sulphide bond reduction, oxidation or isomerization in such proteins [7–10]. It is a 55 kDa protein which contains two mutually homologous active sites, each of which include a pair of catalytically active cystines [11]. Usually, the enzymatic activity is measured by refolding of reduced denaturated ribonuclease in the presence of PDI and a thiol compound. The rate with which the ribnuclease becomes active is a measure of the PDI activity [12]. However, the substrate for this assay is not well defined and involves complex interactions between the enzyme and substrate. It furthermore requires fairly large amounts of PDI as well as ribonuclease. Assays for a direct detection and measurement of the PDI activity would therefore be desirable. Consequently, interchain disulphide bond containing libraries were synthesized in order to identify suitable substrates for PDI. The peptide library was generated on a polyethylene glycol cross-linked polyamide (PEGA) resin [13] which swells greatly in both organic solvents and aqueous buffers [14] and allows biological active proteins up to $\sim$80 kDa [15] to enter into the interior of the beads. This polymer has also been used to characterize the specificity of serine [5] and metalloproteinases [15] in peptide libraries. The library consists of two peptides, one peptide with a randomized sequence and one with a non-randomized sequence, linked by a disulphide bridge. The randomized peptide contains a Cys residue surrounded by random amino acid residues (all the natural amino acids except Cys) and the Abz moiety. This peptide was linked to the PEGA resin. The library was generated by the split synthesis method [2] to yield one peptide compound on each bead. The Met residue was used as a linker so that peptides could be selectively cleaved from the resin by CNBr [16]. The non-random peptide contains an activated Cys residue in a defined sequence and a Tyr(NO_2) residue. The interchain disulphide bridge was formed between the generated library linked to the PEGA resin and the peptide containing the activated Cys residue with the Tyr(NO_2) moiety. Preparation of libraries and substrates containing the donor–acceptor pair, the Abz and Tyr(NO_2), residues and defined disulphide bridges will be discussed.

MATERIALS AND METHODS

General Methods

All organic solvents were purchased from Labscan Ltd (Dublin, Ireland). Suitable protected N^{α}-Fmoc-amino acids and *p*-((α-Fmoc-amino)-2,4-dimethoxybenzyl)-phenoxyacetic acid (Rink-amide-linker) were purchased from Nova Biochem (Switzerland), TBTU and Dhbt-OH from Fluka (Switzerland), NEM from Merck (Germany). The PL-PEGA resin was from Polymer Laboratories (UK). The molecular weights of the peptides were determined, using matrix-assisted laser desorption time-of-flight mass spectroscopy (MALDI-MS), recorded on a Lasermat 2000 (Finnigan Mat). Analytical HPLC was performed using a Waters RCM 8 × 10 module and with a Deltapak C-18 column (19 × 300 mm). Preparative HPLC was performed on a Hitachi L-6250 Preparative Intelligent Pump using a Deltapak C-18 column (25 × 200 mm). The solvent system for both analytical and preparative HPLC was buffer A, 0.1% TFA in water, and buffer B, 0.1% TFA in 90% acetonitrile–10% water and UV detection was at 215 or 280 mm for analytical and only 215 nm for preparative. The gradient for analytical HPLC (1 ml/min); a linear gradient of 0–100% buffer B over 50 min and preparative HPLC (10 ml/min); a linear gradient of 0–60% buffer B over 70 min unless otherwise stated. The PEGA-beads were analysed under an Optical Star Fluorescence Microscope with a 320 nm band pass filter for the excitation and a 410 nm low pass filter for the detection of emitted light.

Solid-phase Synthesis: General

The synthesis of peptides using the PEGA resin [13] was performed by the plastic syringe technique [17]. The side-chain amino acid protecting groups were *t*Bu, for Ser, Thr and Tyr; trityl (Trt) for Asn, Gln and His; O-*t*Bu for Asp and Glu; Trt, *t*Bu, Npys or *p*Npys for Cys; *tert*-butyloxycarbonyl (Boc) for Lys and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. Fully protected N^{α}-Fmoc amino acid OPfp esters (3 mol equiv.) [18, 19] were coupled in DMF with the addition of a catalytic amount of Dhbt-OH whereas Fmoc-Cys(Npys)-OH (3 mol equiv.) was coupled after *in situ* activation with TBTU [20]. The N^{α}-Fmoc group was removed by 20% piperidine in DMF. After completion of the synthesis, the resin was washed with DMF (× 5) and DCM (× 5) and dried before the deprotection and cleavage. The peptides were side chain-deprotected and cleaved simultaneously from the Rink-linker by treatment with 95% TFA for 2 h and the resin was then rinsed with 95% acetic acid (× 4). For peptides containing a single thiol, the cleavage was mediated by a mixture of 95% TFA and triisopropylsilane (3 mol equiv.) to scavenge the long-lived trityl carbonium

ion. TFA and acetic acid were removed under reduced pressure and after precipitation with diethyl ether the crude product was purified by preparative HPLC. Peptides that were attached to the resin via Met were cleaved by CNBr [21]; a solution (50 μl) of CNBr in 0.1 N HCl (20 mg/ml) was added to 10–20 beads. The mixture was kept in the dark for 16 h at room temperature. Water (50 μl) was added to the solution and the reaction was stopped by freezing and lyophilizing to dryness. The peptides were analysed by analytical RP-HPLC and MALDI-MS.

Attachment of the Rink-linker. Rink-amide-linker [22] (3 mol equiv.) was dissolved in DMF and cooled to 0 °C and TBTU (3 mol equiv.) and NEM (6 mol equiv.) were added. After 10 min, the solution was added to the PEGA resin (1 mol equiv.) which had been swelling in DMF (1 h). The flask was gently shaken for 2 h and the resin was filtered and rinsed with DMF (× 6) and DCM (× 5), respectively. The unreacted amino groups were capped by the addition of 10% acetic anhydride in DMF (20 min).

Attachment of the Methionine Residue. Methionine (3 mol equiv.) was coupled to the PEGA resin (1 mol equiv.) in DMF as its Pfp-ester and in the presence of Dhbt-OH (1 mol equiv.).

Solid-phase Peptide Synthesis Using the Plastic Syringe Technique. The syntheses of the compounds **1, 2, 3, 6**, the precursor of compound **10** as well as the compounds **13a–26a** were performed using a disposable plastic syringe (without piston) fitted with a sintered Teflon filter (pore size 70 μm) and connected to a waste bottle through a two-way valve. The PEGA resin (200 mg to 1.5 g) containing either the Rink-linker or the Met residue was placed in the syringe. The Fmoc-group was removed with 20% piperidine and the resin was rinsed with DMF (× 8). The first amino acid (3 equiv.) was then coupled in DMF in the presence of Dhbt-OH to either the Rink-linker or the Met residue and the acylation time was visually followed by a decrease in the yellow colour of the resin (30 min). The resin was rinsed with DMF (× 8). The following amino acid residues were coupled in a similar fashion. The protected fluoroescence probe Boc-Abz-ODhbt (3 equiv.) [11,12] was coupled in DMF while the quencher Fmoc-Tyr (NO_2)-OH (3 equiv.) [11,12] was activated with TBTU and NEM in DMF prior to coupling.

Abz-Ala-Cys-Ala-NH_2 1. Compound **1** was prepared as described above using a PEGA resin (115 mg, 0.52 mmol/g). The Cys residue was protected with a trityl group. The crude product was purified by semi-preparative RP-HPLC. Compound **1** was pure according to analytical HPLC (t_R 14.1 min), obtained as a solid (10 mg, 44%) and characterized by MALDI-MS; m/z 380.5 $(M+H)^+$ ($C_{16}H_{22}N_5O_4S_1$, requires M, 380.1).

Abz-Ala-Cys-Ala-Met-PEGA resin 2. Compound **2** was prepared as described above using a PL-PEGA resin (167 mg, 0.2 mmol/g). The Cys residue was protected with a trityl group. A few beads were collected and cleaved by CNBr. The peptide was pure according to analytical RP-HPLC (t_R 18.6 min).

Cys(Npys)-Ala-Tyr(NO_2)-Ala-NH_2 3. Compound **3** was prepared as described above using a PEGA resin (75 mg, 0.52 mmol/g). The Cys residue was protected with a Npys group. The crude product was purified by semi-preparative RP-HPLC. Compound **3** was pure according to analytical HPLC (t_R 24.5 min), obtained as a solid (6.8 mg, 28%) and characterized by MALDI-MS; m/z 624.7 $(M+H)^+$ ($C_{23}H_{27}N_8O_9S_2$, requires M, 623.1).

Abz-Ala-Cys(Cys-Ala-Tyr(NO_2)-Ala-NH_2)-Ala-NH_2 4. Compound **1** (1.06 mg, 2.7 μmol) was dissolved in phosphate buffer, pH 7.5 (1 ml) and compound **3** (3.2 μmol, 2 mg) was added. The mixture was stirred for 2 h at room temperature and the progress of the reaction was monitored at RP-HPLC (t_R 20.2 min). Compound **4** was obtained in a 67% yield (1.6 mg) after purification by semi-preparative RP-HPLC and was characterized by MALDI-MS; m/z 853 $(M+H)^+$ and 875 $(M+Na)^+$ ($C_{34}H_{47}N_{11}O_{11}S_2$, requires M, 851).

Abz-Ala-Cys(Cys-Ala-Tyr(NO_2)-Ala-NH_2)-Ala-Met-PEGA resin 5. Compound **2** (40 nmol) was suspended in H_2O/DMF (1 : 1) (60 μl) and compound **3** (0.1 mg, 160 nmol) was added. The progress of the disulphide bond formation was monitored under a fluorescence microscope and was completed within 2 h. Few beads were collected and cleaved by CNBr. Compound **5** was obtained in >95% yield based on HPLC. The peptide was analysed by analytical RP-HPLC (t_R 21.4 min) and characterized by MALDI-MS; m/z 952.3 $(M+H)^+$ ($C_{38}H_{53}N_{11}O_{14}S_2$, requires M, 951.3).

Ala-Ser-Ala-Cys-Ala-Tyr(NO_2)-Ala-NH_2 6. The precursor of compound **7** was prepared as described above using a PEGA resin (350 mg, 0.45 mmol/g).

The Cys residue was protected with a trityl group. The crude product was purified by semi-preparative RP-HPLC. Compound **6** was pure according to analytical HPLC (t_R 18.3 min), obtained as a solid (53.7 mg, 49%) and characterized by MALDI-MS; *m/z* 725 $(M + Na)^+$ ($C_{27}H_{40}N_9O_{11}S_1$, requires M, 698.3).

Ala-Ser-Ala-Cys(pNpys)-Ala-Tyr(NO_2)-Ala-NH_2 7. The unprotected Cys residue in compound **6** was converted to Cys(*p*Npys) [23] to give compound **7**. A solution of DTNP (11.9 mg, 38.4 μmol) in acetic acid : water (3 ; 1, V/V) (3 ml) was added to compound **6** (6.7 mg, 9.6 mmol). The solution was stirred for 6 h at room temperature and the progress of the reaction was monitored at analytical PHLC. The solvent was removed under reduced pressure and the crude product was purified by semi-preparative HPLC. Compound **7** was pure according to analytical HPLC (t_R 24.2 min), obtained as a solid (4 mg, 49%) and characterized by MALDI-MS; *m/z* 877 $(M + Na)^+$ ($C_{32}H_{42}N_{11}O_{13}S_2$, requires M, 852.2).

Abz-Ala-Cys(Ala-Ser-Ala-Cys-Ala-Tyr(NO_2)-Ala-N-H_2)-Ala-NH_2 8. Compound **1** (0.6 mg, 1.58 μmol) was dissolved in ammonium acetate buffer (1 M pH 6.2, 700 μl) and compound **7** (1.3 mg, 1.58 μmol) was added. Themixture was stirred for 2 h at room temperature and the progress of the reaction was monitored at RP-HPLC (t_R 20.8 min). Compound **8** was obtained in a 55% yield (0.95 mg) after purification by semi-preparative RP-HPLC and was characterized by MALDI-MS; *m/z* 1081 $(M + H)^+$ ($C_{43}H_{62}N_{14}O_{15}S_2$, requires M, 1079.1).

Abz-Ala-Cys(Ala-Ser-Ala-Cys-Ala-Tyr(NO_2)-Ala-N-H_2)-Ala-Met-PEGA resin 9. Compound **2** (40 nmol) was suspended in ammonium acetate buffer (1 M, pH 6.2, 100 μl) and compound **7** (0.3 mg, 352 nmol) was added. The progress of the disulphide bond formation was monitored under UV illumination in a stereo-microscope and was completed within 3.5 h. A few beads were collected and cleaved by CNBr. Compound **9** was obtained in $>95\%$ purity based on HPLC. The peptide was analysed by analytical RP-HPLC (t_R 21.9 min) and characterized by MALDI-MS; *m/z* 1164.6 $(M + H)^+$ ($C_{47}H_{66}N_{14}O_{17}S_2$, requires M, 1163.3).

Ala-Tyr(NO_2)-Cys(pNpys)-Ala-NH_2 10. The precursor of compound **10** was prepared as described above using a PEGA resin (400 mg, 0.45 mmol/g). The Cys residue was protected with a trityl group. The crude product was pure according to analytical HPLC (t_R 16.5 min) and obtained in a quantitative yield (87 mg). The unprotected Cys residue was converted to Cys(*p*Npys) to give compound **10** using the procedure by Rabanal *et al.* [23]. Compound **10** was pure according to analytical HPLC (t_R 24.1 min) obtained in a 55% yield (23 mg) and characterized by MALDI-MS; *m/z* 626.4 $(M + H)^+$ ($C_{23}H_{28}N_8O_9S_2$, requires M, 624.5).

Synthesis of inter-chain disulphide bridge containing libraries 11 and 12. The Fmoc-Met-PEGA resin was prepared as described above using a PL-PEGA resin (1 g, 0.2 mmol/g for compound **11** and 1.5 g for compound **12**). The resin was then transferred to a 20 column library generator [24]. The libraries, which contain all the natural amino acids except Cys, were generated by the split synthesis method [2] and were synthesized as previously described (3 equiv. of each amino acid in the presence of Dhbt-OH is used in each column) [24]. The Cys residue was protected with a trityl group and was coupled as its OPfp ester [18, 19]. At the amino terminus, Boc-Abz-ODhbt in DMF was coupled to the resin. The resin-linked libraries were deprotected by a mixture of 95% TFA (5 ml/g resin) and TIS (3 mol equiv.) for 2.5 h in a syringe followed by a wash with 95% acetic acid. A few beads were collected, cleaved by CNBr and analysed by MALDI-MS. Multiple peaks were detected in the mass range of 950–1150 for the precursor of library **11** and 1100–1300 for the precursor of **12**. The resin-linked library precursor of library **11** was treated twice with a solution of compound **10** (10 mol equiv.) in ammonium acetate buffer (1 M, pH 6.2)/DMF (1 : 1) (4 ml/51.6 μmol free peptide) under argon overnight. Similarly, library **12** was prepared using compound **7** (10 mol equiv.). For library **11**, the progress of the disulphide bond formation was monitored under fluoroescence UV illumination in a stereo-microscope. For library **12**, many beads were still fluorogenic under the fluoroescence stereo-microscope after treatment with compound **7**. Therefore, some of the beads were cleaved by CNBr and analysed by MALDI-MS. Multiple peaks were detected in the mass range of 1800–2000 indicating complete disulphide bond formation. In addition, a few beads of the library **11** were analysed by MALDI-MS after CNBr cleavage and multiple peaks in the mass range of 1600–1800 were detected.

Synthesis of the compounds 13b–26b. The different peptide fragments utilized for the preparation of the compounds **13b–26b** were prepared by MCPS [18]

as described above for the syringe method using a PEGA resin (333 mg, 0.3–0.4 mmol/g). The Cys residue was protected with a trityl group. The crude peptide was pure according to analytical RP-HPLC (t_R 30.5 min (**13a**), 28.0 min (**14a**), 29.1 min (**15a**), 30.2 min (**16a**), 28.5 min (**17a**), 30.2 min (**18a**), 30.7 min (**19a**), 26.1 min (**20a**), 24.9 min (**21a**), 30.1 min (**22a**), 27.9 min (**23a**), 30.0 min (**24a**), 28.8 min (**25a**) and 28.0 min (**26a**)), obtained as solids (average yield 50%) and were characterized by MALDI-MS (see Table 1). The peptide fragments (5 μmol) were each dissolved in DMF (0.6 ml) and compound **10** (3.1 mg, 5 μmol) was added. The solutions were stirred overnight at room temperature and the progress of the reaction was monitored at RP-HPLC (t_R 29.3 min (**13b**), 27.3 min (**14b**), 28.5 min (**15b**), 29.3 min (**16b**), 26.3 min (**17b**), 28.6 min (**18b**), 28.7 min (**19b**), 28.2 min (**20b**), 26.2 min (**21b**), 28.3 min (**22b**), 27.2 min (**23b**), 28.6 min (**24b**), 28.6 min (**25b**) and 28.8 min (**26b**)). The compounds **13b–26b** were obtained in an average yield of 51% after purification by semi-preparative RP-HPLC and were characterized by MALDI-MS (see Table 1).

RESULTS AND DISCUSSION

The Basic Considerations in the Design of the Library

The enzyme PDI [7] catalyses disulphide rearrangements in proteins and consequently assists their folding [8]. In the present work, peptide libraries have been synthesized for the characterization of PDI. The libraries contained the fluoroescent Abz group and the quenching chromophore Tyr(NO_2) incorporated in two peptide chains separated by a disulphide bridge. During enzymatic disulphide bond reduction, the Tyr(NO_2)-containing peptide chain is cleaved from the resin-bound Abz-containing peptide. Substrates are visualized as beads with increased fluorescence and are then identified by amino acid sequence analysis. It has previously been demonstrated [6] that the donor–acceptor pair must be less than eight amino acid residues apart when separated by peptide bonds to obtain complete quenching. Thus, two libraries were designed to investigate the influence of the donor–acceptor distance via disulphide bridges. In one library (**11**, Figure 4), the distance between the donor–acceptor pair was four amino acid residues in addition to the S–S bond. The cysteine in the resin-bound peptide of this library was surrounded by four random amino acids. In a second library (**12**, Figure 4), the donor–acceptor distance was increased to six amino acid residues in addition to the disulphide bridge.

Synthesis of Compounds 4, 5, 8 and 9

To establish the chemistry for the library, two interchain disulphide bound heterodimers were synthesized as free peptide amides (Figure 1(A), **4** and **8**) as

Table 1 Analysis of Compounds **13–26** and Their Precursors (Abz-GX_1C(R)X_2 X_3X_4MG-NH_2, where **a**, R = H or **b**, R = Ala-Tyr(NO_2)-Cys-Ala-NH_2)

Compound	Molecular formula	Molecular mass (calcd/found)[a]	Compound	Molecular formula	Molecular mass (calcd/found)[a]
13a	$C_{41}H_{59}N_{10}O_{11}S_2Na$	955.2/955.9	**13b**	$C_{59}H_{83}N_{16}O_{18}S_3Na$	1423.6/1424.7
14a	$C_{38}H_{62}N_{11}O_9S_3Na$	936.2/937.1	**14b**	$C_{56}H_{86}N_{17}O_{16}S_4Na$	1304.6/1406.7
15a	$C_{37}H_{59}N_{10}O_{10}S_3Na$	923.1/923.2	**15b**	$C_{55}H_{83}N_{16}O_{17}S_4Na$	1391.6/1392.0
16a	$C_{35}H_{55}N_{10}O_9S_3Na$	878.1/880.0	**16b**	$C_{53}H_{79}N_{16}O_{16}S_4Na$	1347.5/1347.6
17a	$C_{39}H_{51}N_{12}O_{11}S_2Na$	951.0/952.1	**17b**	$C_{57}H_{75}N_{18}O_{18}S_3Na$	1419.5/1421.2
18a	$C_{39}H_{64}N_{11}O_9S_2Na$	818.1/918.1	**18b**	$C_{57}H_{88}N_{17}O_{16}S_3Na$	1386.6/1388.9
19a	$C_{42}H_{68}N_{11}O_9S_2Na$	958.2/959.7	**19b**	$C_{60}H_{92}N_{17}O_{16}S_3Na$	1426.7/1427.9
20a	$C_{40}H_{64}N_{13}O_{11}S_3$	999.2/999.7[b]	**20b**	$C_{58}H_{88}N_{19}O_{18}S_4Na$	1490.7/1491.0
21a	$C_{36}H_{56}N_{13}O_{10}S_2$	895.0/896.1[b]	**21b**	$C_{54}H_{80}N_{19}O_{17}S_3Na$	1386.5/1388.0
22a	$C_{39}H_{62}N_{11}O_{11}S_2Na$	948.1/948.7	**22b**	$C_{57}H_{86}N_{17}O_{18}S_3Na$	1416.6/1417.1
23a	$C_{41}H_{58}N_{11}O_9S_2Na$	936.0/936.2	**23b**	$C_{59}H_{82}N_{17}O_{16}S_3Na$	1404.6/1405.0
24a	$C_{42}H_{63}N_{12}O_9S_2Na$	967.2/967.5	**24b**	$C_{60}H_{87}N_{18}O_{16}S_3Na$	1435.6/1436.2
25a	$C_{38}H_{62}N_{11}O_9S_3Na$	936.2/937.7	**25b**	$C_{56}H_{86}N_{17}O_{16}S_4Na$	1404.6/1405.4
26a	$C_{38}H_{62}N_{11}O_9S_3Na$	936.2/937.1	**26b**	$C_{56}H_{86}N_{17}O_{16}S_4Na$	1404.6/1404.7

[a] Peptides calculated as $[peptide + Na]^+$ ions.
[b] Peptides calculated and detected as $[peptide + H]^+$ ions.

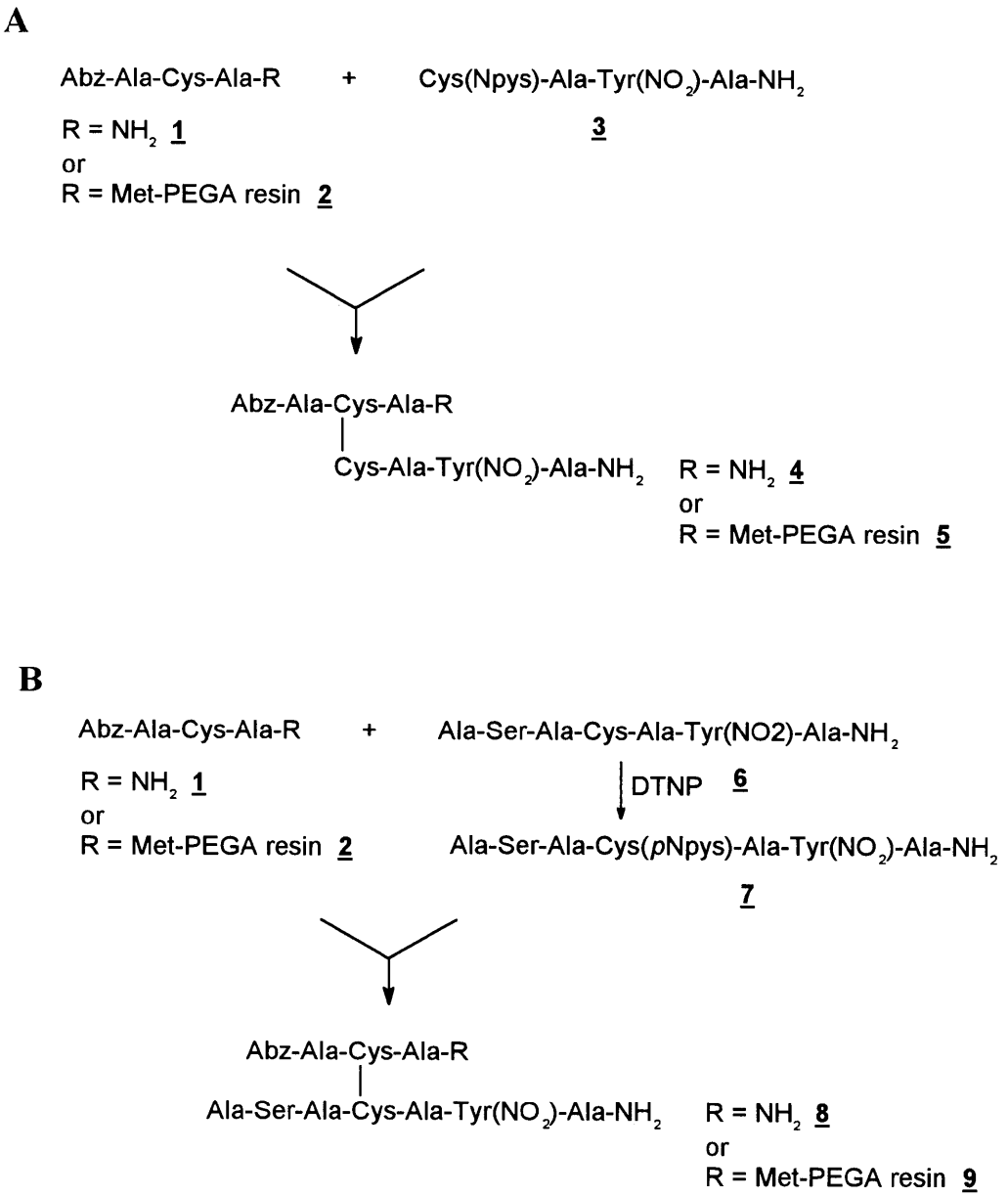

Figure 1 Synthetic scheme for preparation of two disulphide bonded heterodimers in solution (**4** and **8**) and on solid phase (**5** and **9**), respectively. The disulphide bond is formed by reaction of a thiol with the Npys derivative of Cys (A) and *p*Npys derivative of Cys (B).

well as linked to the solid-phase (Figure 1(B), **5** and **9**). Compounds **1, 2, 3** and **6** which were used to generate the two heteodimers were synthesized by stepwise solid-phase synthesis [25] using Fmoc chemistry [26, 27] on a PEGA resin [13]. The couplings were performed with Fmoc-amino acid-Pfp esters with the addition of Dhbt-OH as an acetylation catalyst whereas the Abz moiety was incorporated as a Dhbt-ester and Tyr(NO_2) moiety as its free carboxylic acid using the TBTU method [20]. A Rink-linker was used to yield free peptides upon the TFA cleavage. To obtain peptides which were still attached to the resin after TFA treatment, Met was utilized as a linker. Compounds **1, 2, 3** and **6** were synthesized by the plastic syringe technique [17] and the functional side chains were fully protected. For compounds **1, 2** and **6**, the side chain of Cys intended to generate a free thiol after TFA cleavage

was protected with trityl. In order to form an interchain disulphide bond between the two peptide chains, the Cys residue on compounds **3** and **7** were protected with Npys [28] (Figure 1(A)) and *p*Npys [23] (Figure 1(B)), respectively, suitable for nucleophilic attack by the free thiol of the deprotected Cys residue. The Cys derivative, Cys(Npys), was commercially available as a Boc amino acid and was readily incorporated into the stepwise solid-phase strategy. However, the activated/protected Cys residue can only be incorporated at the *N*-terminus of the peptide sequence. This is because the Npys group is not compatible with the Fmoc/*t*Bu strategy owing to its instability under strong basic conditions. A peptide containing an internal Cys(Npys) residue could be formed by reacting thiol-containing peptides with Npys-Cl [29], but disulphenyl chlorides are moisture-sensitive and can react with the indole ring of tryptophan [30]. Alternatively, the pNpys group, which is an isomer of Npys and retains the same essential properties, can be introduced by adding the disulphide reagent DTNP [23] instead of disulphenylchloride. The peptides were side-chain deprotected with 95% TFA containing 3 mol equiv. triisopropyl silane (TIS) since deprotection of Cys(Trt) is reversible in absence of suitable scavengers [31]. Simultaneously, compound **1, 3** and **6** were cleaved from the resin support and eluted as a major peak in analytical HPLC, giving an average yield of 41% of the purified product.

Characterization of the purified compound **1, 3** and **6** by MALDI-MS showed the correct mass. After CNBr-cleavage, compound **2** showed only one major peak in analytical HPLC. Compound **6** was treated with DTNP to activate its Cys residue and compound **7** was formed. The progress of the reaction was monitored by analytical HPLC and the expected product, as confirmed by MALDI-MS, was formed in 49% yield after purification. The first heterodimer (Figure 1(A), **4**) was formed in phosphate buffer from the reaction between a 1 : 1 ratio of peptide **1** and the S-activated/protected compound **3**. The interchain disulphide bond was formed within 2 h monitored by analytical HPLC (Figure 2(A)) and the yield of the expected product was 67% after purification. The same heterodimer was also synthesized linked to the solid phase (Figure 1(A), **5**) from compound **2** on a PEGA resin in excess of compound **3**. The reaction was performed in H_2O/DMF (1 : 1) and through monitoring the reaction under a fluoroescence microscope, the hetero-disulphide bond formation was completed in 2 h. Analytical HPLC of heteodimer **5** after CNBr cleavage provided only one major peak

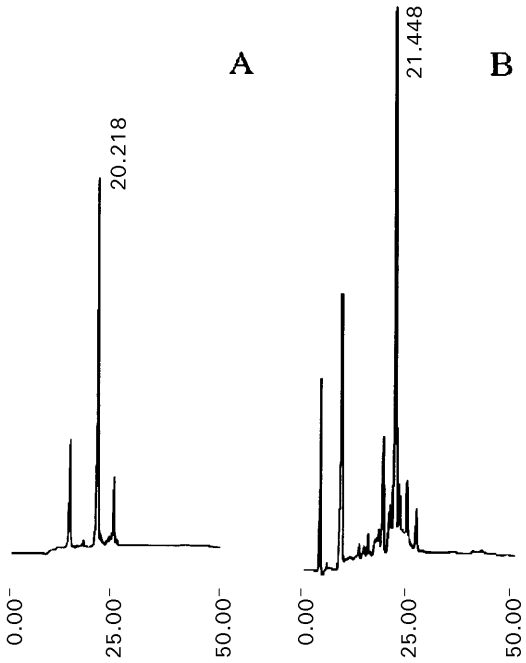

Figure 2 C-18 reversed-phase HPLC profiles of the crude heterodimer **4** (A) (from the reaction between peptides **1** and **3**) and the crude heterodimer **5** (B) after CNBr-cleavage.

(Figure 2(B)) and characterization by MALDI-MS confirmed the expected mass. Similarly, the second heterodimer was formed in solution (Figure 1(B), **8**) and on solid phase (Figure 1(B), **9**), respectively, from compound **1** or **2** by the reaction with compound **7**, and were obtained as a major peak at analytical HPLC (Figure 3(A) and 3(B)) in 55% and $>95\%$ yields, respectively.

Synthesis of the Libraries 11 and 12

In library **11** (Figure 4), the Abz probe was separated from the disulphide bridge by a distance of two amino acids. The Cys residue of this peptide chain was surrounded by one randomized amino acid at the N-terminal side and three randomized amino acids at the C-terminal side. The precursor of library **11** was readily assembled on the PEGA resin in a 20 column library generator using the split synthesis method. After removal of the side-chain protecting groups of the resin-linked library with 95% TFA containing 3 equiv. TIS, compound **10** was covalently attached through a disulphide bond using the Cys(pNpys) activation strategy to generate the heterodimer library **11**. Analysis of a few beads from the

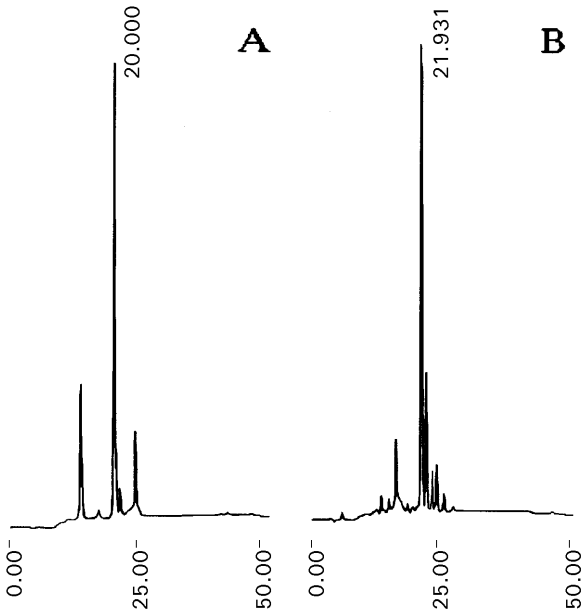

Figure 3 C-18 reversed-phase HPLC profiles of the crude heterodimer **8** (A) (from the reaction between peptides **1** and **7**) and the crude heterodimer **9** (B) after CNBr-cleavage.

precursor of library **11**by MALDI-MS after CNBr-cleavage gave multiple peaks in the mass range 950–1150. The progress of the disulphide bond formation was monitored by viewing the disappearance of fluoroescence on the beads under a fluoroescence microscope. The interchain disulphide bond formation of the heterodimer library **11** was also confirmed by MALDI-MS and provided multiple peaks in the range 1600–1800. To increase the number of randomized residues, peptide library **12** (Figure 4) was also synthesized. The distance between the fluorogenic donor–acceptor pair was six amino acid residues in addition to the disulphide bridge. In library **12**, the cysteine was flanked by three randomized amino acids on each side. The precursor library was generated as described above and characterization after CNBr-cleavage by MALDI-MS showed multiple peaks in the mass range 1100–1300. The formation of the heterodimeric library **12** could not be monitored under the fluoroescence stereo-microscope since some of the beads were still fluoroescent after repeated treatment with compound **7**. A few beads were cleaved by CNBr and analysis by MALDI-MS provided peaks in the range of 1800–2000 which demonstrated that the disulphide bridges had been formed. Based on this result, the optimal distance between the fluorogenic donor and acceptor pair in the library has to be four amino acid residues or less in addition to the disulphide bond to be suitable for enzymatic screening.

Ala-Tyr(NO_2)-Cys(*p*Npys)-Ala-NH_2

10

Abz-Gly-X-Cys-XXX-Met-PEGA-resin
|
Ala-Tyr(NO_2)-Cys-Ala-NH_2

11

Abz-XXX-Cys-XXX-Met-PEGA resin
|
Ala-Ser-Ala-Cys-Ala-Tyr(NO_2)-Ala-NH_2

12

R
|
Abz-Gly-X_1 -Cys-$X_2X_3X_4$-Met-Gly-NH_2

a: R = H
b: R = Ala-Tyr(NO_2)-Cys-Ala-NH_2

	X_1	X_2	X_3	X_4
13	Ser	Thr	Phe	Ile
14	Ala	Met	Lys	Val
15	Ala	Thr	Met	Ile
16	Gly	Met	Ala	Leu
17	Tyr	Ser	Gly	His
18	Gly	Leu	Lys	Leu
19	Pro	Ile	Lys	Leu
20	Arg	Val	Met	Glu
21	Pro	Ala	Arg	Ser
22	Ile	Asn	Ile	Thr
23	Gly	Phe	Lys	Pro
24	Leu	Pro	His	Leu
25	Gly	Met	Lys	Leu
26	Met	Lys	Leu	Gly

Figure 4 The amino acid sequences of compound **10**, the peptide libraries **11** and **12** and the synthetic substrates **13–26** suitable for PDI.

Synthesis of Substrates 13b–26b

Substrates **13b–26b** to be used for the characterization of PDI were synthesized in solution with no difficulty similar to the preparation of heterodimer **8** (Figure 1(B)). The crude **13a–26a** were obtained in an average yield of 50% and were characterized by MALDI-MS (Table 1). The interchain disulphide bond formations were carried out in DMF since the peptide segments were poorly soluble in aqueous solution. The reactions were visually monitored by the intense yellow colour due to the released 5-nitro-2-pyridinethiol. Analytical RP-HPLC of the crude substrates **13b–26b** provided only one major peak and the average yield after separation by semi-preparative RP-HPLC were 51%. The hetero-disulphide bond formation was confirmed by MALDI-MS (Table 1). The enzymatic screening of the library using human PDI [4] expressed from *Escherichia coli*, as well as the specificity and studies of PDI using synthetic substrates, will be discussed elsewhere [4].

CONCLUSIONS

In this work, model hetero-dimeric peptides linked together via disulphide bridges were synthesized by disulphide bond formation both in solution and on solid phase and the chemistry for the preparation of PDI substrate libraries have been established. These contained defined disulphide bonds for characterization of the specificity of PDI. Two libraries containing a defined interchain disulphide bridge and the Abz/Tyr(NO_2) energy transfer donor–acceptor pair were synthesized. For one library, the S–S bond was surrounded by four randomized amino acid residues and the distance between the donor and acceptor was four residues in addition to the disulphide bridge, whereas the second library contained six randomized amino acids surrounding the S–S bond and had the donor–acceptor pair distance of six amino acids in addition to the disulphide bridges. Only the library with the shorter distance between the donor and acceptor was found to give adequate internal quenching. Also, substrates found by screening the library with hPDI were synthesized in solution in an average yield of 51%.

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