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ORIGINAL ARTICLE

Investigation of the automated solid-phase synthesis of a 38mer peptide with difficult sequence pattern under different synthesis strategies

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Abstract Difficult peptides are a constant challenge in solid-phase peptide synthesis. In particular, hydroxyl amino acids such as serine can cause severe breakdowns in coupling yields even several amino acids after the insertion of the critical amino acid. This paper investigates several methods of improving synthesis yields of difficult peptides including the use of different resins, activators and the incorporation of a structure-breaking pseudoproline dipeptide building block both alone and in combination with each other.

Keywords Difficult peptides · Pseudoproline dipeptides · Fmoc solid-phase synthesis · ChemMatrix resin

Introduction

The synthesis on a solid-phase medium such as a resin or membrane is a standard method for the preparation of peptides and small proteins. To be successful, a synthesis requires almost quantitative coupling yields at each reaction step which can only occur when the reagents have fast and free access to the growing peptide chain on the solid phase (Merrifield and Littau 1968). If a complete coupling is not possible, the reaction yield dramatically decreases with the number of couplings (White and Chan 2000). Peptides that show a significant drop in the yield on distinct coupling steps, or over the time of the entire synthesis process, are called "difficult peptides". Common difficulties include the association of the growing peptide chain with its solid support, or with itself, by formation of hydrogen bonds, hydrophobic interactions or β-sheet formation (Pillai and Mutter 1981; Narita et al. 1985). Such peptides often show characteristic sequence patterns; for instance, the insertion of single amino acids such as serine or threonine can cause difficulties in couplings. These effects may occur 5–6 couplings after the insertion of the causal amino acid (Bedford et al. 1992). Statistically, the likelihood of a coupling issue increases with the length of the growing peptide chain. Changing solvents (Hyde et al. 1992) using chaotropic salts (Klis and Stewart 1990) or the addition of detergents (Zhang et al. 1994) to change the coupling environment usually provides limited to no effects; however, the right choice of coupling reagents has been shown to be an important factor in the degree of synthesis success (McNamara et al. 2000). In recent synthesis strategies the greatest effects came from the insertion of structure-breaking building blocks such as pseudoproline dipeptides (White et al. 2004; Abedini and Raleigh 2005), *O*-acyl isopeptides (depsipeptides) (Carpino et al. 2004; Coin et al. 2006), amino acids with Hmb/Dmb-protected α-amino function (Clippingdale et al. 1999; Cardona et al. 2008), as well as a combination of those strategies (Sampson et al. 1999; Goncalves et al. 2009). Additionally, the use of pseudoproline dipeptide building blocks (PDBBs) and aspartic acid or asparagine-containing dipeptide building blocks with Hmb/Dmb-protected α-amino function (e.g. Fmoc–Asp(OtBu)–(Dmb)Gly–OH) has been proven to reduce aspartimide formation significantly (Quibell et al. 1994; Zahariev et al. 2006; Ullmann et al. 2012; Wang et al. 2012). Furthermore, an application of microwave heating (Pedersen et al. 2012) with or without the use of structure-breaking building blocks has been investigated with promising results

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(Rizzolo et al. 2011; Hussein et al. 2013; Harris et al. 2013) even though the application of heat could also decrease the synthesis yield in some cases (Roodbeen et al. 2012; Echalier et al. 2013). Another effective methodology is the use of a resin with a low functionality per volume which can be achieved by low functionalization of the dry resin or by strong swelling of the polymer in the reaction solution.

In this publication, we investigate the use of a PDBB in combination with different activators and resins and its influence on difficult peptides. The application of PDBBs in a peptide synthesis was first introduced by Mutter and co-workers (Haack and Mutter 1992; Wöhr et al. 1996). As a model peptide we selected the α-helical peptide K5, a 38mer with the five times repeat of the sequence KVSALKE. This peptide was designed by De Crescenzo and co-workers (De Crescenzo et al. 2003) for the investigation of interactions between the two strands of heterodimeric coil-coiled structures. Pillai and Mutter (Pillai and Mutter 1981) described that helical and β-sheet structures could affect the peptide synthesis by aggregation within the growing peptide chains. Here we present our results of the investigation of the synthesis of peptide K5 under different coupling conditions.

Materials and methods

Chemicals

All used amino acids were protected according to Fmoc protection strategy (Fields and Noble 1990). Derivatives and the activators were obtained from GL Biochem (Shanghai, China) and EMD Biochemicals/Novabiochem (Billerica, MA, USA). The PDBB Fmoc–Val–Ser($\psi^{Me,Me}$pro)–OH was purchased from EMD Biochemicals/Novabiochem. All other solvents, eluents and reagents were obtained from VWR (Mississauga, ON, Canada), Fisher Scientific (Ottawa, ON, Canada) or Sigma-Aldrich (St. Louis, MO, USA).

To investigate the influence of the resin types the peptides were synthesized on four different resins:

- Rink amide-AM resin (GL Biochem, Shanghai, China): a high-density polystyrene (PS) resin + 1 % divinylbenzene (DVB), loading capacity: 0.54 mmol/g.
- TentaGel S AM (Intavis, Cologne, Germany): a polyethylene glycol (PEG)-modified low-density resin (Coin et al. 2007), loading capacity: 0.23 mmol/g.
- Rink amide-CLEAR resin (Peptides International, Louisville, KY, USA): a highly cross-linked PEG resin with high swelling behaviour (Kempe and Barany 1996), capacity: 0.49 mmol/g.
- Rink amide-ChemMatrix resin (PCAS BioMatrix, Saint-Jean-sur-Richelieu, Quebec, Canada): a cross-linked PEG resin with strong swelling behaviour (de la Torre et al. 2007; García-Martín et al. 2006a, capacity: 0.46 mmol/g.

General peptide synthesis conditions

Fmoc–His(Trt)–OH and Fmoc–Phe–OH were dissolved in *N*-Methylpyrrolidone (NMP) at a concentration of 0.6 M. All other amino acid derivatives and activators were dissolved in *N*,*N*′-Dimethylformamide (DMF) at a concentration of 0.6 M. A mixture of 45 % 4-methylmorpholine (NMM) in DMF was used as a base. The syntheses were carried out using a MultiPep peptide synthesizer (Intavis, Cologne, Germany).

Synthesis strategy A

For coupling of the first ten amino acids, 400 µl of the solutions of the protected amino acids were mixed with 430 µl of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl) uronium tetrafluoroborate (TBTU) solution and 130 µl of the NMM mixture. The reaction mixture was delivered to a batch of swollen resin with 50 µmol capacity and let to sit for 15 min at which 400 µl DCM was added. After 5 min, the reaction mixture was renewed followed by a second application of DCM 15 min after the delivery of the reaction solution. After another 5 min, the resin was washed with DMF several times and treated with capping solution [2 % acetic anhydride + 2 % *N*-ethyldiisopropylamine (DIPEA) in DMF] twice for 5 min. In preparation of the next amino acid coupling, the resin was washed with DMF and treated with 20 % piperidine/DMF twice at 5 min intervals which was then followed by several DMF washing steps. The coupling of amino acids 11–20 was performed in a similar fashion to the first coupling procedure except the reaction time before the addition of DCM was extended to 20 min and the repeat of the coupling was carried out using a reaction mixture with 1-[Bis(dimethylamino)methylene]-*1H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) instead of TBTU. Additionally, the piperidine treatment was extended to 6 min. For the coupling of amino acid 21–30, the coupling procedure was performed three times, twice with TBTU and once with HATU. The treatment time with piperidine was extended to 8 min. For further couplings, the coupling was carried out once with TBTU and twice with HATU activation.

Synthesis strategy B

The procedure was identical to synthesis strategy A with the exception that the activator TBTU was completely substituted by HATU.

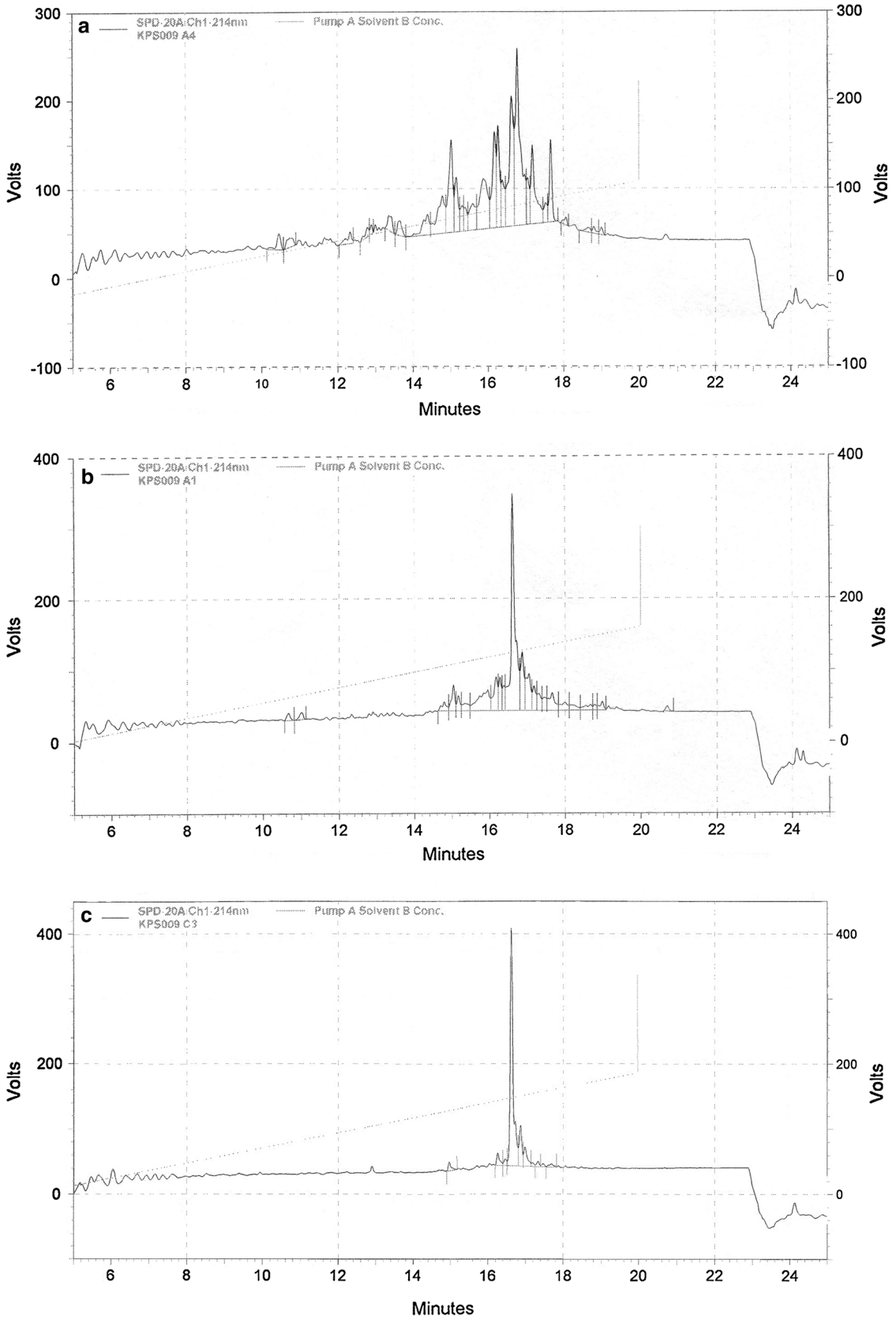

Fig. 1 HPLC images of the synthesis of the long peptide K5 under different conditions: **a** Rink Amide resin and synthesis strategy A; **b** ChemMatrix resin and synthesis strategy A; **c** ChemMatrix resin, use of PDBB and synthesis strategy B

The cleavage of the peptides from resin and the simultaneous deprotection was performed using trifluoroacetic acid (TFA) with 4 % triisopropylsilane (TIPS), 3 % dithiothreitol (DTT) and 1 % water.

Analytics

The peptide purity was tested by analytical reversed-phase (RP) HPLC. Water (+0.1 % TFA; eluent A) and acetonitrile (+0.1 % TFA; eluent B) were used as eluents. 10 µl of the sample solution was injected into an LC-20 System (Shimadzu, Kyoto, Japan). The HPLC analysis was carried out using C-18 RP-HPLC columns (Prosphere HP C18, 300A, 5 µm, Alltech, Deerfield, IL, USA, and Viva C18, 300A, 5 µm, Restek, Bellefonte, PA, USA) with a linear gradient (0 % B–50 % B in 20 min) followed by a 5 min wash with 95 % B and final equilibration with 100 % A for another 5 min at a constant flow of 1 ml/min.

MALDI-ToF–MS analysis was performed using Voyager DE-Pro Workstation (Applied Biosystems, USA) at the Proteomics Core Facility at the University of British Columbia. The peptides were presented as a mixture of 1.0 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in water/acetonitrile (1:1; +0.1 % TFA) mixed with 0.5 µl stock solution of the peptide in the same solvent.

Combination of synthesis conditions

Poor synthesis results of peptide K5 (CGGKVSALKEKVSALKEKVSALKEKVSALKE) (De Crescenzo et al. 2003) that were encountered using the standard synthesis strategy A with common Fmoc-amino acid derivatives on high-loaded Rink amide-AM resin were the initial issue for this investigation (see Figs. 1, 2). In consideration of further investigations for comparison purposes, we added a C-terminal beta-Ala to the original K5. Despite that modification we would still call that peptide K5 in this publication.

Since the peptide K5 consists of a repeated pattern, motif KVSALKE, we investigated the influence of the sequence pattern itself; therefore, we reduced the length of the peptide to only the sequence motif with the additional N-terminal linker sequence CGG and an additional beta-Ala as it is also present in peptide K5. We called this peptide K5a.

To improve the accessibility of the growing peptide chain during the synthesis, we synthesized the peptides K5a and K5 on the different resins that are listed above. These resins were used in both synthesis strategies A and B.

Additionally, to avoid the formation of structures which might affect the coupling, we investigated the effect of the use of a VS-PDBB on the corresponding position(s) during the peptide synthesis for K5a and K5 with synthesis strategies A and B on all four resin types.

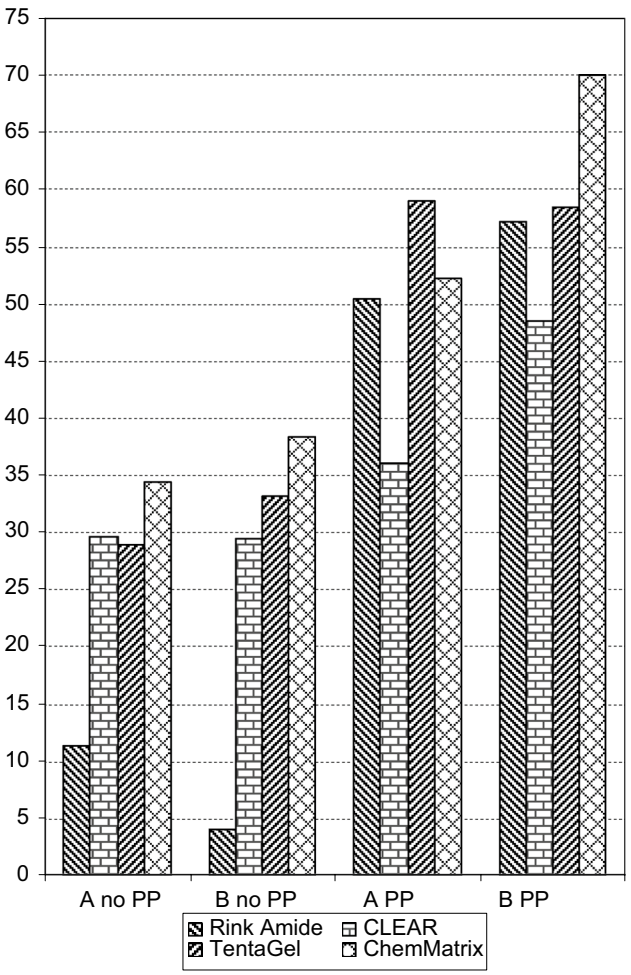

Fig. 2 Purities of the crude long peptide K5 with and without PDBB (*PP*, *no PP*) on different resin and with different synthesis strategies (*A*, *B*)

Results and discussion

The initial synthesis (strategy A, Rink amide-AM resin) yielded a very low amount of the desired product (Figs. 1, 2). It was hard to find the corresponding molecular weight (MW). The highest measured major signal was at a MW of 4,020.6 Da (MW_{calc} = 4,084.8 Da) which relates to the capped peptide missing the final Cys (MW_{calc} = 4,023.6 Da). K5a was synthesized under the same synthesis conditions and the resultant crude product contained only partially the correct peptide (Fig. 3) (MW_{anal} = 1,061.9 Da; MW_{calc} = 1,061.2 Da). The major side product was also a capped peptide without the N-terminal

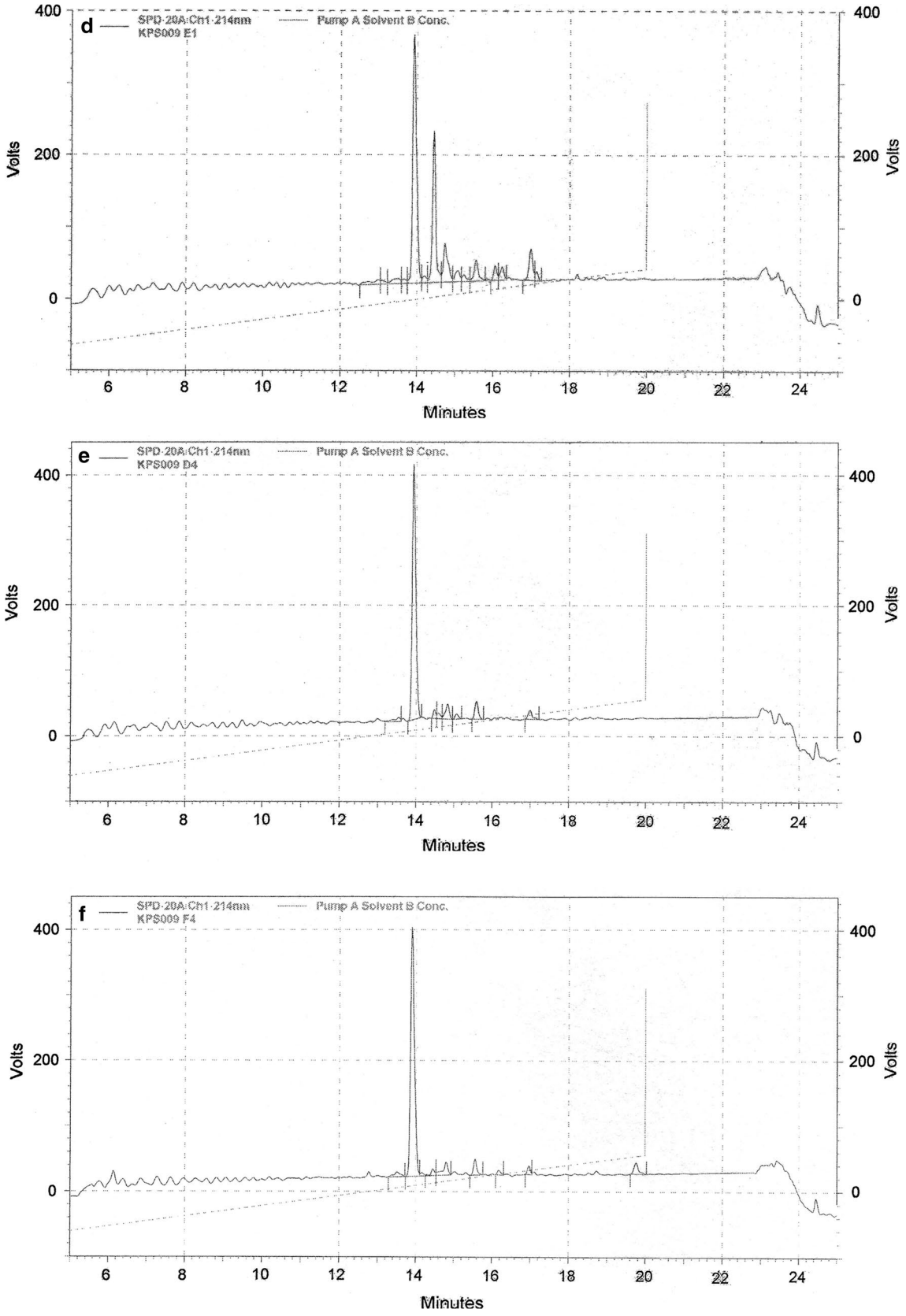

Fig. 3 HPLC images of the synthesis of the short peptide K5a under different conditions: **d** Rink Amide resin and synthesis strategy A; **e** ChemMatrix resin and synthesis strategy A; **f** ChemMatrix resin, use of PDBB and synthesis strategy B

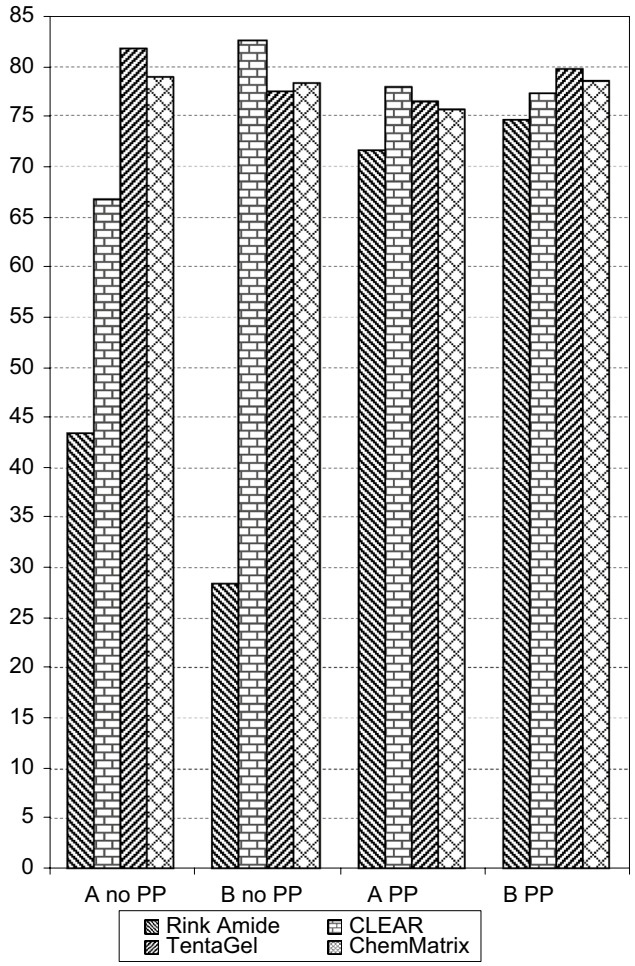

Fig. 4 Purities of the crude short peptide K5a with and without PDBB (*PP*, *no PP*) on different resin and with different synthesis strategies (*A*, *B*)

cysteine ($MW_{anal} = 1{,}000.8$; $MW_{calc} = 1{,}000.1$ Da). Substitution of TBTU by HATU (synthesis strategy B) decreased the coupling quality in the synthesis of peptide K5a as well as K5 (Figs. 2, 4). In both cases of K5a synthesis, the breakdown of coupling efficiency could be observed at the fifth coupling after the insertion of a serine residue.

To avoid the formation of structures which might affect the coupling, we used a Fmoc–Val–Ser($\psi^{Me,Me}$pro)–OH on the corresponding position(s) using synthesis strategy A and B. The insertion of that PDBB showed a significant improvement of the quality of the crude products K5a and K5 (Figs. 2, 4).

For further investigation, we used three other resins to improve the accessibility of the growing peptide chain. While TentaGel S AM has a general low capacity which results in a lower peptide density per volume and a higher peptide accessibility, CLEAR resin and ChemMatrix resins showed a much greater swelling that lead to a lower density after swelling. In comparison to Rink amide-AM resin, the use of all other three resins improved the yield dramatically. Interestingly, the use of CLEAR resin and VS-PDBB for the synthesis of the long peptide K5 with synthesis strategies A and B showed a reduction in the yield in comparison to the synthesis on Rink amide-AM resin under same conditions (Figs. 2, 4).

While the synthesis of the short peptide K5a on TentaGel S AM and ChemMatrix resin already showed a superior performance without the use of the pseudoproline dipeptide building block, the use of CLEAR resin on synthesis strategy A without using a pseudoproline dipeptide building block was less effective. In most cases, impurities were caused by incomplete deprotection of the peptide.

The use of all three PEG-containing resins significantly increased the yield of the long K5 in comparison to the performance of high-density PS based without PEG (Rink amide-AM resin) (Figs. 2, 4). On all resins the performance could be improved by the use of Fmoc–Val–Ser($\psi^{Me,Me}$pro)–OH. In the case of the Rink amide-AM resin the yield increased by tenfold. In most cases, the complete use of HATU (synthesis strategy B) in addition to the use of Rink amide-AM resin led to an improvement. The highest yield on peptide K5 was achieved by the use of ChemMatrix resin in combination with the incorporation of the pseudoproline dipeptide building block applying synthesis strategy B (Fig. 1). Although the performance of TBTU alone was not tested for the long peptide K5, results indicate that in most cases of syntheses of the peptides K5a and K5 the activation with TBTU is not as effective as HATU.

Conclusions

During Fmoc solid-phase peptide synthesis the presence of serine in peptide sequences can lead to severe breakdown in coupling efficiency around the fifth amino acid after the incorporation of the serine. Classical PS/DVB resins with high functional density have shown to be ineffective with difficult peptides, whereas, the use of PEG-based or PEG-modified resins improves yields significantly. In short peptides, the use of such resins could even lead to an elimination of side reactions. For longer peptides or more complex difficult peptides with multiple potential critical hydroxyl amino acids, the substitution of critical amino acids by corresponding PDBBs is highly beneficial. A combination of using PDBBs with PEG-based or PEG-modified resins can improve the synthesis further. The best combination has been proven to be the use of ChemMatrix resin and PDBB in combination with HATU as sole activator confirming similar findings by other research groups (García-Martín et al. 2006b; Northfield et al. 2010; Vernieri et al. 2014).

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Conflict of interest The authors declare that they have no conflict of interest.

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