Substrate Specificity of the Streptococcal Cysteine Protease*

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The streptococcal pyrogenic exotoxin B (SpeB) is an important factor in mediating Streptococcus pyogenes infections. SpeB is the zymogen of the streptococcal cysteine protease (SCP), of which relatively little is known regarding substrate specificity. To investigate this aspect of SCP function, a series of internally quenched fluorescent substrates was designed based on the cleavage sites identified in the autocatalytic processing of SpeB to mature SCP. The best substrates for SCP contain three amino acids in the nonprimed position (i.e. ALK in P3-P2-P1). Varying the length of the substrate on the primed side of the scissile bond has a relatively lower effect on activity. The highest activity (kcat/KM = 2.8 ± 0.6 (10^6 × m^-1 s^-1)) is observed for the pentamer 3-amino-benzoin acid-AIKAG-3-nitrotyrosine, which spans subsites S2 to S1′ on the enzyme. High pressure liquid chromatography and mass spectrometry analyses show that the substrates are cleaved at the site predicted from the autoprocessing experiments. These results show that SCP can display an important level of endopeptidase activity. Substitutions at position Pn of the substrate clearly indicate that the S3 subsite of SCP can readily accommodate substrates containing a hydrophobic residue at that position and that some topological preference exists for that subsite. Substitutions in positions Pn, P1, and P1′ had little or no effect on SCP activity. The substrate specificity outlined in this work further supports the similarity between SCP and the cysteine proteases of the papain family. From the data regarding the identified or proposed natural substrates for SCP, it appears that this substrate specificity profile may also apply to the processing of mammalian and streptococcal protein targets by SCP.

Streptococcus pyogenes belongs to group A streptococci responsible for suppurative infections of the pharynx ("strep throat"), skin (impetigo, cellulitis), and subcutaneous soft tissues (necrotizing fasciitis, myositis) (1, 2). S. pyogenes is also the cause of more systemic infections that can result in toxic shock-like syndrome, rheumatic fever, glomerulonephritis, and scarlet fever (1, 2). A growing concern over the rising incidence of the antibiotic resistant strains of this bacterium (3) intensified studies aimed at resolving the mechanism of infection and prompted the search for new treatments.

S. pyogenes produces a number of virulence factors, of which the most studied is the exotoxin known as the streptococcal pyrogenic exotoxin B (SpeB)(4, 5). SpeB is the 39-kDa protein precursor of a cysteine protease (EC 3.4.22.10). It is a highly conserved protein, which unlike the other exotoxins (SpeA and SpeC) is encoded by the chromosomal DNA. There is significant evidence for an important role of SCP in mediating invasion of the endothelium (6). The mature protease was shown to cleave human fibrinectin and to degrade vitronectin (7), to process prointerleukin-1-β to active interleukin (8), to release biologically active kinins from H-kinninogen (9), and to liberate M protein and C5a peptidase from the streptococcal cell surface (10). In vivo studies have shown that purified SCP is lethal to mice (4). In more recent studies Łukomska et al. (6, 11) and Burns et al. (12) showed that in the absence of the functional speB gene, infection could be controlled by the host’s immunological system. All mice infected with a wild type pathogen died, whereas most of those challenged with S. pyogenes carrying the altered speB gene survived.

In microbial cultures of S. pyogenes, SpeB is secreted to the culture medium. The proenzyme has only one cysteine residue that can be oxidized readily (13). Under a reducing environment (DTT, β-mercaptoethanol) the protein catalyzes its own processing. In a previous study we characterized the in vitro stepwise autoprocessing of SpeB and identified five cleavage sites (14). As shown by Kagawa et al. (15), four of these sites occupy accessible loops in the pro-region, explaining the bimolecular autoprocessing of the proenzyme (14, 15). Three of the four accessible sites have the same amino acids (Ile-Lys) in positions P3-P1 (16). The hydrolysis of Abz-peptides corresponding to the SpeB autoprocessing sites occurs at the same peptide bond as expected from thezymogen autoprocessing studies. The substrate Abz-AIKAGAR, homologous to the first autoprocessing cleavage site, showed the highest kcat/KM value (7.7 10^3 × m^-1 s^-1). Therefore, this sequence was chosen to design a series of IQF substrates. The IQF substrates are composed of a varying length peptide flanked at the N terminus by a fluorophore, o-aminobenzoic acid, and at the C-terminal end by 3-nitrotyrosine or 4-nitrophenylalanine that acts as a

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1 The abbreviations used are: SpeB, streptococcal pyrogenic exotoxin B (SCP zymogen); SCP, streptococcal cysteine protease; Abz, 3-aminobenzoic acid; β-Boc, β-butylloxycarbonyl; Bz-, benzoyl; Chg, cyclohexylglycine; DTT, dithiothreitol; F(4NO2), 4-nitrophenylalanine; Fmoc, N-9-fluorenylmethoxycarbonyl; HPLC, high pressure liquid chromatography; IQF substrate, internally quenched fluorogenic substrate; MS, mass spectroscopy; Nle, norleucine; Phg, phenylglycine; pNA, p-nitroanilide; Tbg, tert-butyglycine; Y(3NO2), 3-nitrotyrosine.
fluorescence quencher. The use of extended IQF substrates will allow the determination of the endopeptidase activity of SCP and permit a more systematic evaluation of substrate specificity. In this study, several IQF substrates were prepared and tested against the mature streptococcal cysteine protease.

**EXPERIMENTAL PROCEDURES**

**Materials**—All IQF and p-nitroanilide substrates were synthesized using amino acid derivatives purchased from Bachem Inc. (Torrance, CA) and resins from Novabiochem (La Jolla, CA). DTT was purchased from Roche Molecular Biochemicals and E-64 from IAF Biochem International Inc. (Laval, Quebec, Canada).

**Production and Purification of Native Streptococcal Cysteine Protease**—The S. pyogenes strain B220 was used for production and purification of SpeB. The proenzyme was purified from the culture broth in a 50 mM phosphate, pH 7.0) gradient in 50 mM phosphate, pH 7.0. Fractions of pure zymogen were collected and dialyzed against 20 mM bis-Tris propane, 100 mM NaCl gradient, 20 mM acetate buffer, pH 5.0) followed by the hydrophobic chromatography on butyl-Sepharose (1.6–0.4 mM ammonium sulfate gradient in 50 mM phosphate buffer, pH 7.0). Fractions of pure xymogen were concentrated and dialyzed against 20 mM bis-Tris propane buffer, pH 7.0. To prepare mature SCP, the xymogen was incubated for 3 h at 37 °C in the presence of 10 mM DTT. Excess DTT was removed from the processed SCP by filtration on Sephadex G25 and stored at 4 °C.

**Synthesis of IQF Substrates**—All quenched fluorescent substrates were synthesized using the Fmoc strategy and prepared in the C-terminal amide form as described previously (17). The peptides were synthesized in a stepwise manner using 4-2′, 4′-dimethoxyphenyl-Fmoc-aminoethylphenoxy resin (Rink acid resin). N-Methyl pyrrolidone was used as a solvent and diisopropycarbodiimide/N-hydroxysuccinimide as condensing agents. For protection of Nα-Fmoc groups, 20% piperidine in N-methyl pyrrolidone was employed. The following side chain-protecting groups were used: trityl-asparagine, glutamine, and histidine, t-butylyl-aspartic acid, glutamic acid, serine, threonine, and tyrosine; 2,2,5,7,8-pentamethylchroman-6-sulfonyl-arginine; and t-butoxycarbonyl-lysine. Peptides were deprotected and cleaved from the resin using a mixture of trifluoroacetic acid, thioanisole, m-cresol, ethanediol, H₂O (80:5:5:5:5, v/v) at 20 °C for 2 h. Crude peptide products were precipitated and washed with ethyl ether, redissolved in 50% acetic acid, and purified by reverse-phase HPLC (using a Vydac C18 column and a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid). The purity and identity of the peptides were confirmed by HPLC and mass spectrometry (Sciex API IIIE triple quadrupole, Thornhill, Ontario, Canada).

**Synthesis of Bz-AIK-pNA**—This substrate was synthesized by reacting H-AIK-Boc-pNA with benzoic acid/dicyclohexylcarbodiimide (18). H-AIK-Boc-pNA was obtained from Fmoc-AIK-Boc-pNA, which was in turn obtained by coupling of Fmoc-A-OB and H-IK-Boc-pNA. The Fmoc group was removed with 20% piperidine, and the Boc blocking group with trifluoroacetic acid. Crude pNA substrate preparations were precipitated with diethyl ether, redissolved in 20% acetic acid, and purified by reverse-phase HPLC. The purity and identity of the substrate were confirmed by HPLC and mass spectrometry.

**Enzyme Assays**—SCP activity was determined in a 96-well plate format by monitoring the changes in fluorescence upon substrate hydrolysis using the Cytofluor Multwell Plate Reader (PerSeptive Biosystems, MA) equipped with excitation (365 nm) and emission (440 ± 10 nm) filters. Because the experiments were carried out at (S) < Km, the kcat/Km values were calculated by dividing initial velocities by enzyme and substrate concentrations. The concentration of SCP was estimated spectrophotometrically using the extinction coefficient E₂₈₀nm = 45 244 μM⁻¹ cm⁻¹ (19) and verified by active site titration with E-64 (20). For the IQF substrates, calibration curves were obtained as described previously (21). An important inner filter effect can be observed at high substrate concentrations, but this effect is not significant at substrate concentrations lower than 20 μM and did not interfere with the experiments carried out at 10 μM substrate. The kinetic characterization of SCP using Abz-peptides (non-quenched) as substrates was done as described previously (14). The influence of pH on the endopeptidase activity of SCP was obtained by determining kcat/Km values for selected IQF substrates in the pH range of 3 to 10. The calibration curves were obtained for the entire pH range used in the study, to take into account the pH dependence of Abz fluorescence. The kcat/Km for ionization of 3-nitrotyrosine in Abz-AIKAG-Y(3NO₂) was determined for a pH range from 4 to 10, and the pH dependence was fitted to a nonlinear regression of the data to the Michaelis-Menten equation.

**HPLC/MS Analysis of Cleavage Sites**—Samples containing 25 μM IQF substrate were hydrolyzed up to 50% with SCP in 50 mM phosphate, 2 mM p-mercaptoethanol, pH 7.0. The tryptic reaction was stopped by adding trifluoroacetic acid to a final concentration of 0.5%. A 50-μl sample was injected onto a C18 silica gel column and separated with a linear gradient (0.05–10% acetonitrile in water, both containing 0.1% trifluoroacetic acid). Intact substrate and hydrolysis products were detected using UV (210 nm) and fluorescence detectors (Waters, Mississauga, Ontario, Canada). The HPLC peaks were collected, freeze-dried, redissolved in 5% acetic acid in 50% acetonitrile, and analyzed by MS. The presence of both H-γ-A3NO₂ and Abz-AIKOH in substrate hydrolysates (see Table II) was also verified by HPLC (retention times 21.3 and 23.5 min, respectively). In most cases only one fluorescent product was observed on the HPLC chromatogram. Any deviation from this pattern is indicated in the text.

**RESULTS**

**Endopeptidase Activity of SCP**—To probe the endopeptidase activity of SCP, internally quenched fluorescent substrates derived from the autoprocessing sites of SpeB were synthesized and tested against mature SCP (Table I). The first IQF substrates synthesized were octamer derivatives of the identified processing sites in SpeB. The results obtained with these substrates show the same pattern of relative activity as obtained earlier with Abz-peptide octamers (14). Hydrolysis of the IQF substrate Abz-SAAIKAG-Y(3NO₂) by SCP displayed the highest kcat/Km value (2.6 ± 0.6 10⁻⁴ μM⁻¹ s⁻¹), and the HPLC and mass spectrometry analyses showed that cleavage occurs at the predicted site (K1 A2). This peptide, corresponding to the first autoprocessing event, was therefore chosen as a template for the substrates used in this investigation.

To define how substrate occupancy affects the endopeptidase activity of SCP, a series of IQF substrates of varied length was first synthesized. The longest substrates contain eight amino acid residues in the peptide portion separating the fluorescence donor from the quencher group. As shown in Fig. 1, they are...
hydrolyzed by SCP with relatively low $k_{\text{cat}}/K_M$ values ($2.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ or lower). The best substrates obtained contain three amino acids in the nonprimed position (i.e. ALK in P$_3$P$_2$P$_1$).

When the unprimed side of the substrate is extended beyond alanine in P$_3$, 6–17-fold decreases in $k_{\text{cat}}/K_M$ are observed. However, the presence of the amino acid residue in P$_4$ is important because a significant decrease in activity results from removal of alanine by Abz in P$_3$ (Fig. 1). On the other hand, varying the length of the substrate on the primed side of the scissile bond has a relatively low effect on activity. The highest activity ($k_{\text{cat}}/K_M = 2.8 \pm 0.6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) is observed for the pentamer Abz-AIKAG-Y(3NO$_2$)$_3$, which spans subsites S$_0$ to S$_1$ on the enzyme. It is important to note that all of the substrates shown in Fig. 1 are cleaved after lysine. Similar effects were noted for other substrates corresponding to the autoprocessing sites reported in Table I. Decreasing the length of the peptide portion of these substrates from octamers to pentamers has no effect on the position of the scissile bond but leads to an overall 10–25-fold increase in $k_{\text{cat}}/K_M$ except for the octamer/pentamer substrates representing the second autoprocessing site, where no change in activity is observed (Table I). The best endopeptidase activity is therefore observed when subites S$_0$ to S$_1$ on the enzyme are occupied by amino acid residues for the substrate. It must be noted that in a previous study it was shown that Z-VR-MCA (benzylloxy carbonyl-l-valine-l-arginine-7-amido-4-methylcoumarin) is a rather poor substrate for SCP (14). Considering the need for a substrate to contain an amino acid residue able to interact in subsite S$_0$ of the enzyme, the peptidyl p-nitroanilide derivative Bz-AIK-pNA was synthesized and tested as a substrate for SCP. The $k_{\text{cat}}/K_M$ for hydrolysis of Abz-AIK-Y(3NO$_2$)$_3$.  

Influence of pH on SCP Activity against the IQF Substrates—The 3-nitrotyrosine group was chosen as the fluorescence quencher to assure a better reduction of basal fluorescence for the uncleaved substrates. Under assay conditions, the signal to background ratio for these substrates ranged from 20 to 40 with the Y(3NO$_2$) residue compared with a ratio of ~4 when using 4-nitrophenylalanine with the more extended substrates (e.g., 6 amino acids). The fact that the quencher possesses an ionizable group (Y(3NO$_2$)) could however present a problem in result interpretation, because this ionization might affect or modulate the rate of substrate hydrolysis. To verify this hypothesis, the pH activity profile for hydrolysis of substrates containing the Y(3NO$_2$) group was determined and compared with that of a corresponding substrate containing the non-ionizable F(4NO$_2$) group as quencher. The results are presented in Fig. 2. The data can be fitted to models that consider that either one or two ionizable group(s) modulates activity. For the substrate Abz-AIKAG-F(4NO$_2$)$_3$, only one pH-dependent transition is observed in the acid range of the profile, with a $pK_a$ of 4.78. A similar sigmoidal pH activity profile with only one $pK_a$ value (4.44) was observed by Kortt and Liu (22) when testing SCP activity against neutral and positively charged acyl-amino acid esters. For the substrate Abz-AIKAG-Y(3NO$_2$)$_3$, in addition to the transition in acidic pH region ($pK_a = 4.70$), the activity is observed to decrease at pH higher than 7, with a $pK_a$ of 7.57 (Fig. 2). This modulation of activity in the pH range 6.5–8.5 is attributed to ionization of the Y(3NO$_2$) group. The $pK_a$ for ionization of the substrate Y(3NO$_2$) group, determined by measuring the influence of pH on absorbance at 430 nm, was evaluated at 7.13. In addition, it must be noted that despite the difference in the pH activity profiles, the $k_{\text{cat}}/K_M$ values determined for Abz-AIKAG-Y(3NO$_2$)$_3$ and Abz-AIKAG-F(4NO$_2$)$_3$ at pH 7.0 are very similar. From the pH activity profiles presented in Fig. 2, it is clear that ionization of the Y(3NO$_2$) group does not interfere with the determination of kinetic ($k_{\text{cat}}/K_M$) parameters.

Evaluation of SCP Specificity with IQF Substrates—Three of the five identified autoprocessing sites in SpeB bear the XIK-X motif (14). In particular, a hydrophobic residue is found in P$_0$ of all five autoprocessing sites (Ile, Tyr, or Val). This suggests that the nature of the residue in position P$_0$ could be important for substrate recognition by SCP. To investigate the effect of the P$_0$ residue on substrate hydrolysis by SCP, Abz-AKKAG-Y(3NO$_2$)$_3$ substrates were prepared with X = Phe, Leu, Asn, Val, Ala, or Gly. The results are presented in Table II. The highest activity was observed for the substrate with isoleucine in P$_0$, which corresponds to the residue found in the consensus
sequence XIK-X'. The substrate with phenylalanine at P2 gave a slightly lower activity. Another hydrophobic residue (Val) also yielded a substrate that was hydrolyzed rapidly by SCP (k_{cat}/K_M 1'-fold lower than for P2 = Ile). A notable exception is leucine, where k_{cat}/K_M was found to be 86-fold lower than observed for the substrate with isoleucine in P2. Lack of detectable enzymatic activity against Abz-ALKAG-OH (data not shown) further supports the preference of SCP for isoleucine over leucine in P2 position. This result is quite interesting, because the two amino acids differ only by side-chain branching. To probe further the effect of side-chain length and branching, four IQF substrates with unnatural amino acids (norleucine (Nle), tert-butylglycine (Tbg), phenylglycine (Phg), and cyclohexylglycine (Chg)) were synthesized. All four substrates showed from 13- (Nle) to 470-fold (Tbg, Chg, and Phg) lower enzymatic activity against Abz-ALKAG-OH (data not shown). As pointed out earlier in this article, the presence of an amino acid residue in the position P3 of IQF substrates appears to be important for SCP activity. To define how the nature of the P3 residue affects specificity, alanine in the control substrate (Abz-AIKAG-OH) was replaced by Val, Leu, Phe, Glu, Lys, and Pro (Table II). No significant effect was observed on the rates of hydrolysis except for the substitution of alanine by proline, which led to a 180-fold decrease in k_{cat}/K_M. However, removal of alanine and shifting Abz to the P3 position led to a 9-fold decrease in k_{cat}/K_M value (Fig. 1). Our data also show no significant effect of P1 substitutions on the second order rate constant, k_{cat}/K_M (Table II).

**DISCUSSION**

SpeB is only one of many virulence factors for *S. pyogenes* infections (2), but the exact mechanism by which it enhances the group A streptococci virulence is not clear. It is believed that during the host-pathogen interaction, the protease cleaves both mammalian (7–9) and streptococcal proteins (10, 23) and that this process, which relies on the endopeptidase activity of the enzyme, is important for invasiveness and protection from the host defense mechanisms. Most of what is known regarding the substrate specificity of SCP comes from early work by Kortt (22) and Gerwin (24), who defined SCP as a good esterase but a poor endopeptidase. To determine the SCP endopeptidase specificity, Gerwin et al. (24) used insulin as a substrate. The bonds cleaved most rapidly were those that had hydrophobic amino acids such as valine, tyrosine, and phenylalanine in position P2. For those major cleavage sites, the P3 position was occupied by either asparagine, glutamic acid, leucine, or phenylalanine. In the same study, the authors tested a series of benzyloxycarbonyl-dipeptides, where the benzyloxycarbonyl residue occupied substrate S2 on the enzyme and the amino acids interacted with
subsites S₁ and S₁'. The \( k_{cat}/K_M \) values for hydrolysis of these substrates were low (40–80 M⁻¹ s⁻¹) and it was concluded that SCP displays poor endopeptidase activity. It must be noted, however, that these substrates, which are not blocked at their C termini, are better suited for carboxypeptidases, and the results indicate that SCP is not a good carboxypeptidase.

Using extended IQF substrates, which span the prime and nonprimed subsites of the enzyme, we have shown that SCP can display an important level of endopeptidase activity. The IQF substrates are hydrolyzed rapidly with \( k_{cat}/K_M \) values as high as \( 10^{3} \times \text{M}^{-1} \text{s}^{-1} \) or more. The highest rate constants were obtained for IQF substrates with three amino acids in the nonprimed positions and with a hydrophobic residue in position P₂. The preference for a hydrophobic residue at that position is in agreement with the findings of Gerwin et al. (24).

An interesting property of SCP is its strong (86-fold) preference for isoleucine over leucine in position P₂. The additional structural diversity in the side chains for the P₂ residues of the substrates, leading to important variations in \( k_{cat}/K_M \) values, indicate that the enzyme displays a topological preference for the P₂ residue in a substrate. The preference of SCP for hydrophobic residues in position P₂ is somewhat similar to that observed for the papain-like cysteine proteases (25, 26). It is also known that position P₁, and in particular P₁', plays a lesser role in defining specificity of the endopeptidase members of the papain-like proteases (e.g. cathepsins K, L, S) (25, 27). SCP is indeed part of the papain superfamily of cysteine proteases (clan CA) and has been predicted to have a three-dimensional structure similar to that of papain (28). At the structural level, this similarity has been confirmed by the determination of the crystal structure of the zymogen, SpeB (15). Despite only 14% sequence identity between SCP and papain, both structures possess similar folds. Superimposition of the protease part of the SpeB structure with that of actinidin shows 44% of Cα atoms aligned within 2Å positional difference. However, in the SpeB zymogen structure, the S₂ subsite appears to be absent compared with papain-like enzymes, and the possibility for alternative substrate binding modes has been considered (15). Even though definite information regarding substrate binding to SCP must await the determination of the crystal structure of the mature enzyme, the substrate specificity outlined in this work would argue for a binding mode similar to that of papain-like enzymes. Differences in the nature of the catalytic residues (i.e. the third member of the “catalytic triad”) and/or in the ionization state of the Cys-His dyad between the two enzymes are reflected in the pH dependence of substrate hydrolysis (only one pH-dependent transition was observed for SCP, whereas papain shows a bell-shaped pH profile).

A notable difference between papain-like enzymes and SCP is the fact that the zymogen SpeB is converted to the mature SCP enzyme following a sequential processing mechanism with the accumulation of distinct intermediate forms (14). Accessibility of the autoproteolytic cleavage sites is probably not responsible for this phenomenon, because four of the five autoprocessing cleavage sites identified are located on accessible loops in the SpeB structure (15). The results of the present study, at first glance, seem to suggest that the sequential reaction is not attributable to kinetic factors, i.e. the IQF substrates corresponding to the autoprocessing sites are cleaved at relatively similar rates. However, it must be noted that this work was done using fully processed SCP. To better understand the molecular basis of the sequential processing, the rates of cleavage of model substrates and/or of the various sites on SpeB by the intermediate forms of SpeB would need to be determined. It is possible that the intermediate forms of SpeB display different catalytic and/or specificity characteristics from mature SCP.

A number of natural substrates for SCP have been identified or proposed. Among the most prominent mammalian protein targets are urokinase plasminogen activator receptor (29), H kininogen (9), fibronectin, vitronectin (7), and the precursor of interleukin 1-β (8). In some cases, the precise location of the cleavage site is known. For human plasma H-kininogen, the initial fast cleavage step by SCP involves hydrolysis at the PFR \( \mathbb{S}^{389} \) S sequence followed by the release of bradykinin (RPPGFSP) from the C-terminal end of the kininogen heavy chain (9). SCP was also shown to cleave the interleukin 1-β precursor in vitro between residues His\(^{115}\) and Asp\(^{116}\), i.e. one amino acid upstream from a caspase-3 cleavage site (8).

In this case, the P₂ and P₃ positions are occupied by valine and histidine, respectively. The SCP has also been implicated in processing of its own surface proteins such as C5a peptidase, M-proteins (10), and streptolysin O (23). Streptolysin O exists in two active forms, of high and low molecular weights. Pinkney et al. (23) showed that transition from the high to the low-molecular weight form is catalyzed by SCP, and a cleavage site was identified as MIK\(^{77}\) L\(^{78}\)A. The biological significance of streptolysin O processing by SCP is not clear, but mutations and chemical modifications of streptolysin O were shown to affect the oligomerization and pore formation properties of this protein (30). It must be noted that several of these studies were performed in vitro only, often using relatively high concentrations of SCP. However, from the data available it appears that the substrate specificity profile identified in this work also applies for the processing of mammalian and streptococcal protein targets by SCP, particularly under conditions in which the enzyme is present in relatively low amounts.

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Endopeptidase Activity of SCP

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Page 44551: In the abbreviations footnote, “Abz, 3-amino- benzoic acid” should be “Abz, 2-aminobenzoic acid.” Also “IQF” should be defined as “internally quenched fluorescent substrate.”

All internally quenched fluorescent substrates were synthesized using Fmoc-2-aminobenzoic acid.