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ORIGINAL ARTICLE

Impact of fluorination on proteolytic stability of peptides: a case study with α-chymotrypsin and pepsin

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Abstract Protease stability is a key consideration in the development of peptide-based drugs. A major approach to increase the bioavailability of pharmacologically active peptides is the incorporation of non-natural amino acids. Due to the unique properties of fluorine, fluorinated organic molecules have proven useful in the development of therapeutically active small molecules as well as in materials and crop science. This study presents data on the ability of fluorinated amino acids to influence proteolytic stability when present in peptide sequences that are based on ideal protease substrates. Different model peptides containing fluorinated amino acids or ethylglycine in the P2, P1′or P2′ positions were designed according to the specificities of the serine protease, α-chymotrypsin (EC 3.4.21.1) or the aspartic protease, pepsin (EC 3.4.23.1). The proteolytic stability of the peptides toward these enzymes was determined by an analytical RP-HPLC assay with fluorescence detection and compared to a control sequence. Molecular modeling was used to support the interpretation of the structure–activity relationship based on the analysis of potential ligand-enzyme interactions. Surprisingly, an increase in proteolytic stability was observed only in a few cases. Thus, this systematic study shows that the proteolytic stability of fluorinated peptides is not predictable, but rather is a very complex phenomenon that depends on the particular enzyme, the position of the substitution relative to the cleavage site and the fluorine content of the side chain.

Keywords Chymotrypsin · Pepsin · Aminobutyric acid · Difluoroethylglycine · Trifluoroethylglycine

Abbreviations

Abz	*o*-Aminobenzoic acid
HPLC	High performance liquid chromatography
Fmoc	Fluorenylmethoxy carbonyl
MD	Molecular dynamics
DIC	Diisopropylcarbodiimide
HOBT	1-Hydroxybenzotriazole
HOAT	1-Hydroxy-7-azabenzotriazole
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
SPPS	Solid phase peptide synthesis

Introduction

Peptides have great potential as drug candidates, provided that a few small limitations can be overcome. They have many advantages over small molecules on the one hand and antibodies on the other hand in terms of affinity, and specificity with respect to their targets. Their use as drugs has been hampered by different factors such as low bioavailability, relatively low solubility, membrane permeability and most profoundly low proteolytic stability (Saffran et al. 1986; Giannis and Kolter 1993; McGregor 2008). Proteolysis, the process by which proteases break

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down peptides and proteins, underlies the basic principle of peptide based drugs. Understanding this principle and the involved mechanisms provides a key strategy to produce peptide or protein-based drugs. Over the past few decades, numerous reports have been published in which a number of strategies employed to combat these limitations are outlined: cyclization of peptides (Hummel et al. 2006; Hruby 2002; Ferrie et al. 2013), obstruction of N- or C-terminal ends (N-acylation, N-pyroglutamate, C-amidation, N-terminal esterification (phosphoester)) or addition of carbohydrate chains (glycosylation: glucose, xylose, hexose), PEGylation (Dasgupta et al. 2002; Lee et al. 2003; Werle and Bernkop-Schnürch 2006; Vlieghe et al. 2010), the introduction of noncanonical amino acids such as D-amino acids, β-amino acids, *N*-methylated amino acids and to lesser extent fluorinated analogues (Łęgowska et al. 2009; Sani et al. 2006; March et al. 2012). In general, the introduction of noncanonical amino acids with newly designed side-chain functionalities serves as a powerful tool to improve kinetic and thermodynamic properties, proteolytic and structural stabilization of peptides and proteins that are not accessible using exclusively the 20 proteinogenic amino acids (Dougherty 2000; Salwiczek et al. 2009a, b; Gottler et al. 2008; Frackenpohl et al. 2001; Koksch et al. 1996a, b).

Due to its unique properties, fluorine has become an important element in various industrial fields like agriculture, materials science, and the pharmaceutical and mining industry (Isanbor and O'Hagan 2006; Filler and Saha 2009). The introduction of fluorine into molecules has led to the improvement of therapeutic agents (Muller et al. 2007; Meng et al. 2008) typically by increasing hydrophobicity and metabolic stability, which in turn leads to improved bioactivity and bioavailability (Böhm et al. 2004). The use of fluorine in the design of artificial amino acids is now being explored in peptide and protein therapeutics, a class of promising agents with high specificity but low metabolic stability (Sato et al. 2006).

Studies on the protease-catalyzed incorporation of different fluoroalkyl-substituted amino acids and their impact on structure, stability and activity of biologically active peptides have shown the importance of fluorine content and the position of fluorination within an amino acid sequence of peptides and proteins (Koksch et al. 1996a, b, 1997; Smits and Koksch 2006; Salwiczek et al. 2009a, 2012).

However, recent investigations of the inclusion of fluorinated amino acids into therapeutic peptides (Meng and Kumar 2007), host defense antimicrobial peptides (Meng et al. 2008), globular proteins (Baker and Montclare 2011), histone acetyltransferase (HAT) tGN5 (Voloshchuk et al. 2009), tripeptide epoxyketone proteasome inhibitor (Geurink et al. 2010) and model peptides (Asante et al. 2013) have indicated that, this incorporation can have controversial effects on activity and stability. Introduction of hexafluoroleucine (a sterically demanding extensively fluorinated amino acid) into the therapeutic peptide, "glucagon-like peptide-1(GLP-1[7-36])" showed protection against regulatory protease (Dipeptidylpeptidase 4, DPP IV) (Meng et al. 2008). On the contrary to these observations, the global incorporation of monofluorinated phenylalanines into histone acetyltransferase (HAT) tGN5 led to reduced proteolytic stability against chymotrypsin (Voloshchuk et al. 2009). Even though elastase has specificity for Ala, the replacement of Ala with TfeGly and DfeGly at different positions within a 10-amino acid model peptide led to proteolytic resistance in two cases while a decreased proteolytic stability was observed only in one case (Asante et al. 2013).

Proteases are found in all organisms and play important roles in (1) physiological processes including digestion, haemostasis, apoptosis, signal transduction, reproduction and the immune response and (2) disease states, such as cancer, viral infection, Alzheimer's disease, inflammatory and cardiovascular disorders (Coughlin 2000). Commercially, proteases are valuable tools in the pharmaceutical and biotechnological industries (Saeki et al. 2007). Proteases are grouped according to their mechanism of catalysis, structural features and common evolutionary origin. Serine and aspartic proteases are two major groups that have been well studied.

α-Chymotrypsin EC 3.4.21.1 is a serine endopeptidase for which the three-dimensional structure, mode of catalysis and substrate specificity have been well characterized. Cleavage of the scissile peptide bond is carried out by a catalytic triad in which the three amino acid residues Ser195, Asp102 and His57 function together as a 'charge-relay' system (Blow et al. 1969). An important part of the catalytic scheme is the formation of an unusually short 'catalytic' hydrogen bond between histidine and aspartate that makes the histidine more basic and aids in the deprotonation of the serine (Derewenda et al. 1994). α-Chymotrypsin cleaves preferably peptide bonds on the C-terminal side of large hydrophobic residues, such as phenylalanine, tyrosine, tryptophan and leucine (Keil 1992; Czapinska and Otlewski 1999; Hedstrom 2002; Polgár 2005).

Pepsin EC 3.4.23.1, an aspartic endopeptidase, is the major digestive protease in the gastric juice of vertebrates and has also been widely investigated since its first crystallization was reported in 1929 by Northrop (Northrop 1930). Aspartic proteases have various mechanisms, but the most commonly used is a general acid–base mechanism which involves the coordination of a water molecule between two highly conserved aspartic acid residues, Asp32 and Asp215 (Antonov et al. 1978, 1981; Suguna et al. 1987; Davies 1990). In such a regime, one

of the aspartic acids acts as a nucleophile and induces hydrolysis between the P1 and P1′ residues of the substrate. Pepsin is known to hydrolyze peptide bonds that connect bulky hydrophobic/aromatic residues, such as Phe-Trp, Phe-Tyr and Phe–Phe (Keil 1992; Fruton 1970; Powers et al. 1977).

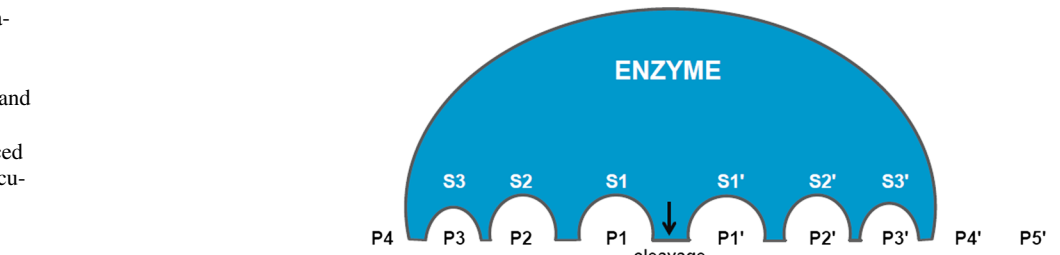
In fact, properties of fluorine in protein environments still remain difficult to predict and more research is required. The current study presents a systematic investigation of the impact of side-chain fluorination on the stability of peptides towards degradation by α-chymotrypsin and pepsin. The main focus of this study is to contribute to the development of "rules of thumb" for the application of fluorine in the design of proteolytically stable variants of clinically relevant peptides.

Materials and methods

Peptide synthesis and purification

All commercially obtained reagents were of synthetic grade and were used as supplied. All peptides were synthesized from the C-terminal to the N-terminal end on a solid support by means of an Fmoc/*tert*-butyl protecting group strategy on preloaded Fmoc-Lysine (Boc) Wang resin using either a Multi-Syntech Syro XP peptide synthesizer (MultiSynTech GmbH, Witten, Germany) or by manual coupling in 10-mL polypropylene reactors. Amino acids and coupling reagents were introduced in four-fold excess and double coupled without capping. The fluorinated amino acids were used in two-fold excess and activated by DIC, HOBt/HOAt to form active esters and the coupling times were extended from 4 to 18 h to ensure completion of the reaction. All peptides (sequences in Fig. 1) were finally N-terminally labeled with *o*-aminobenzoic acid (Abz) to enable photometric detection, as the use of fluorophores within synthetic substrates is a well established method employed to gain fast, sensitive and reproducible quantitative monitoring of proteolytic activity.

The peptides were cleaved from the resin by treatment with 3 mL TFA/TIS/H_2O (95%:5 %:10 µL) for 3 h and precipitated with cold diethyl ether. The crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a smartline system (Knauer GmbH, Berlin), equipped with a smartline manager 5000, two smartline pumps 1000 and a UV detector 2500, with a Phenomenex® Luna C8 (2) column [10-µm particle size, 300 Å pore size, 250 × 21.20 mm (inner diameter)]. A maximum of 20-mg crude peptide per run was purified with a linear gradient of 95 % A to 50 % B over 30 min, where solvent A is 100 % water with 0.1 % TFA (v/v) and solvent B is 100 % acetonitrile with 0.1 % TFA (v/v) at a flow rate of 20 mL/min. Absorbance was recorded at $\lambda_{abs} = 320$ nm. Purity of the peptides was controlled by analytical HPLC and verification of peptide molecular weight was carried out by means of mass spectrometry. All peptides were >96 % pure as monitored by analytical HPLC.



Peptides										
FA	Abz-	Lys	Ala	Ala	Phe	Ala	Ala	Ala	Ala	Lys
P2-TfeGlyFA	Abz-	Lys	Ala	TfeGly	Phe	Ala	Ala	Ala	Ala	Lys
P2-DfeGlyFA	Abz-	Lys	Ala	DfeGly	Phe	Ala	Ala	Ala	Ala	Lys
P2-Abu FA	Abz-	Lys	Ala	Abu	Phe	Ala	Ala	Ala	Ala	Lys
P1'-TfeGlyFA	Abz-	Lys	Ala	Ala	Phe	TfeGly	Ala	Ala	Ala	Lys
P1'-DfeGlyFA	Abz-	Lys	Ala	Ala	Phe	DfeGly	Ala	Ala	Ala	Lys
P1'-AbuFA	Abz-	Lys	Ala	Ala	Phe	Abu	Ala	Ala	Ala	Lys
P2'-TfeGlyFA	Abz-	Lys	Ala	Ala	Phe	Ala	TfeGly	Ala	Ala	Lys
P2'-DfeGlyFA	Abz-	Lys	Ala	Ala	Phe	Ala	DfeGly	Ala	Ala	Lys
P2'-AbuFA	Abz-	Lys	Ala	Ala	Phe	Ala	Abu	Ala	Ala	Lys

Reduced sequence for modeling

Fig. 1 Schematic representation of the enzyme binding pocket named according to Schechter and Berger 1967, and table of the studied peptides. Marked in *grey* are the reduced sequences used for the molecular modeling study

Protease degradation assay

The proteolytic stability of peptides toward α-chymotrypsin (from bovine pancreas, EC 3.4.21.1, 80.0 units/mg) and pepsin (from porcine stomach mucosa EC 3.4.23.1, ≥250 units/mg) (Sigma Aldrich, USA) was analyzed by an analytical RP-HPLC equipped with a fluorescence detector. All peptides employed in the degradation studies were used as the trifluoroacetic acid (TFA) salts obtained after lyophilization. Stock solutions of α-chymotrypsin and pepsin were prepared at concentrations of 0.1 mg/mL in 10-mM phosphate buffer, pH 7.4, and 0.5 mg/mL in 10 mM acetate buffer, pH 4.0, respectively. Peptides were prepared as 20-μmol/mL stocks in DMSO and incubated with the respective enzyme at 30 °C with shaking at 300 rpm in a thermomixer over a period of 2 h. Aliquots of 5 μL were removed at fixed time points (0, 15, 30, 60, 90 and 120 min) and either quenched with 95-μL acetonitrile containing 0.1 % TFA, in the case of α-chymotrypsin, or 2 % aqueous ammonia, in the case of pepsin. A monolithic reversed-phase C8 analytical column (Merck's Chromolith® Column, C8 endcapped, 100–4.6 mm) was used to resolve and quantify the products of digestion.

All samples were either immediately subjected to analytical HPLC or frozen down (−20 °C). Detection based on the Abz label was carried out using a fluorescence detector with $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm.

In all cases, the peaks corresponding to the educts (full-length peptides) or the N-terminal fragment (product) were integrated and used to determine the velocity of the reaction. The FA peptide carrying alanine in positions P2, P1′, and P2′ was used as a reference. The enzyme concentration was adjusted to provide a system in which the reference would be degraded by ~40 % after 120 min since, peptide/protein-based drugs have been shown to exhibit less than 1 % oral bioavailability; the ability to improve this to at least 30–50 % will be of much significance (Vincent et al. 1991; Shaji and Patole 2008).

Each fragment cleaved from the full-length peptide was identified by ESI–MS on an Agilent 6210 ESI-TOF (Agilent Technologies, Santa Clara, CA, USA). All experiments were performed in triplicate.

Molecular modeling

The peptide ligands were built in an extended conformation using MOE (Molecular Operating Environment 2011) provided by the Chemical Computing Group (http://www.chemcomp.com). The Protein Data Bank (PDB) (Berman et al. 2000) entry code 4CHA (Tsukada and Blow 1985) was selected as the starting structure for α-chymotrypsin. The protein was prepared for docking with the LigX module (default parameters) as implemented in MOE with a particular emphasis on the enzymatic cavity and the protonation state of the catalytic triad. Docking studies were carried out using the software GOLD 5.1 (Jones et al. 1995, 1997) using a 100 % search efficiency parameter. The active site was determined by selecting all residues within 10 Å around the hydroxyl group of Ser214. A stepwise increasing number of constrains was set until a conformation in agreement with the literature was obtained. Three constraints were required to orient the peptides in a conformation in agreement with the literature (Fujinaga et al. 1995, 2000; Hedstrom 2002): a distance range from 2 to 5 Å between (1) the backbone CO of Ser214 (protein) and backbone NH of Phe at position P1 (ligand), (2) the backbone NH of Gly193 (protein) and the backbone CO of Phe at position P1 (ligand), (3) the backbone CO of Phe41 (protein) and the backbone NH of the P2′ position (ligand). The PDB entry 1PSA (Chen et al. 1992) was selected as the starting structure for pepsin. The enzyme was prepared for docking with MOE (Molecular Operating Environment 2011) and the water molecule involved in the hydrolysis mechanism but absent from the crystal structure was manually added between the two carboxylic acids Asp32 and Asp215. Docking studies were also carried out using GOLD 5.1 with a 100 % search efficiency parameter and activated toggle + spin options for the water molecule. The active site was determined by selecting all residues within 6 Å around the ligand, pepstatin A, co-crystallized within the pepsin binding cleft. With pepsin, only one constraint was sufficient to generate a peptide orientation in agreement with the literature: a distance range from 1 to 3 Å between the backbone CO of Gly34 (protein) and the backbone NH in position P2 (ligand). After docking, all valid poses were minimized using the MMFF94 force field as implemented in LigandScout version 3.1 (Wolber and Langer 2005; Seidel et al. 2010; Wolber et al. 2006). LigandScout was also used for visualization and analysis of all docking results.

Results

Structure and design

The substrate specificity of both enzymes relies on mainly large hydrophobic residues, such as Phe, Trp and Tyr at the P1 position (Keil 1992). Therefore, a positive control peptide (FA peptide: name based on amino acid residues at the P1 and P1' positions, Phe and Ala, respectively) was designed with a central phenylalanine residue in the P1 position. This design also ensures both that the incorporation of the nonproteinogenic amino acids will not generate a new cleavage site, and that the protease-mediated

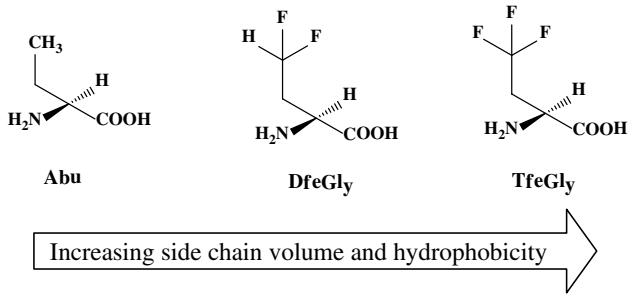


Fig. 2 Structures of the nonproteinogenic amino acids (*S*)-ethylglycine, or aminobutyric acid (Abu), (*S*)-4,4-difluoroethylglycine (DfeGly) and (*S*)-4,4,4-trifluoroethylglycine (TfeGly) used in this study

hydrolysis of the peptides used in this study will be site specific. The sequence of the FA peptide is Abz-Lys-Ala-Ala-Phe-Ala-Ala-Ala-Ala-Lys, as illustrated in Fig. 1. Because the substrate binds to the enzyme's active site in an extended conformation, the alanines in positions P3, P3′, and P4′ act as spacers. Lysines were introduced at the ends of the peptides to enhance solubility, and *o*-aminobenzoic acid (Abz) was used as a fluorescence label. Each alanine residue at the P2, P1′ or P2′ position was singly replaced by the amino acid in question, as shown in Fig. 2. The fluorinated amino acids trifluoroethylglycine (TfeGly) and difluoroethylglycine (DfeGly) differ in their degree of fluorination and hence are expected to place different steric demands on the enzyme's binding pocket. Additionally, such fluoroalkyl side chains have been shown to polarize neighboring C–H bonds with sometimes appreciable effects on intermolecular noncovalent interactions (Salwiczek et al. 2009a, b). Aminobutyric acid (Abu) was also included in this study to distinguish between effects relating to fluorine and those relating to the hydrocarbon chain itself. This strategy led to the synthesis of nine FA variants carrying nonproteinogenic residues in positions that are keys for the recognition of the substrate by the protease.

Protease stability of peptides

The stability of the peptides towards hydrolysis by the proteases was determined by an analytical RP-HPLC assay with fluorescence detection. The use of HPLC for analyzing cleavage products is a well-established method which offers a fast, sensitive and reproducible way to monitor the cleavage reaction (Ferrie et al. 2013; Aguilar 2004; Hua and Huang 2010; Shrimpton et al. 2000).

Each peptide was incubated with each enzyme and the products were quantified and characterized by means of the chromatography software (EZChrom Elite Version 3.1.7), based on peak areas and mass spectrometry. Figure S1 (supporting information) shows the time course of one of the peptides in the presence of α-chymotrypsin and Fig. 3 summarizes the results of all the digestion experiments.

Molecular modeling analysis

To gain insight into the interaction between the peptide ligand and the enzyme binding site, the three FA peptide series were further investigated by molecular modeling. Each peptide/protease combination was subjected to molecular docking using GOLD (Jones et al. 1995, 1997). This software implements a genetic algorithm to represent the rotational, translational and torsion angles of a molecule to explore the conformational space of a ligand within an enzyme binding site. Such an approach remains challenging with peptide structures that have multiple rotatable bonds. To decrease the number of atoms and bonds for the calculation, each sequence was built from the P2 to the P2′ unit, with the carboxy-terminus and the amino-terminus methylated (Me). The substrates were then constructed according to the following short sequence: MeHN-P2-P1-P1′-P2′-Me. For the 10 peptide variants (Fig. 1), docking conformations were generated within the α-chymotrypsin and pepsin enzymatic cavities. The poses were considered valid when the phenylalanine P1-residue was oriented towards the hydrophobic S1 pocket of the enzyme and the backbone of the peptide was stabilized in agreement with previously published studies (Fujinaga et al. 1995, 2000; Hedstrom 2002), as summarized in Table 1. Our hypothesis was that a peptide well stabilized in the active site in silico would be an indication of good enzyme recognition and faster degradation.

Discussion

α-Chymotrypsin

In the molecular modeling study, a majority of the docking poses generated for each peptide displayed a conformation highly stabilized by the interactions reported in Table 1.

These results show excellent complementarity between the residues from P2 to P2′ of the ligand and their corresponding protein subsites S2 to S2′. Moreover, the side chain of Ser195 is always ideally oriented between the P1 and P1′ positions which are in agreement with the key role of this residue in the enzymatic mechanism of α-chymotrypsin. These conformations were therefore considered to be a solid basis to support structural arguments regarding the ligand-enzyme interactions studied here, and their influence on proteolytic stability.

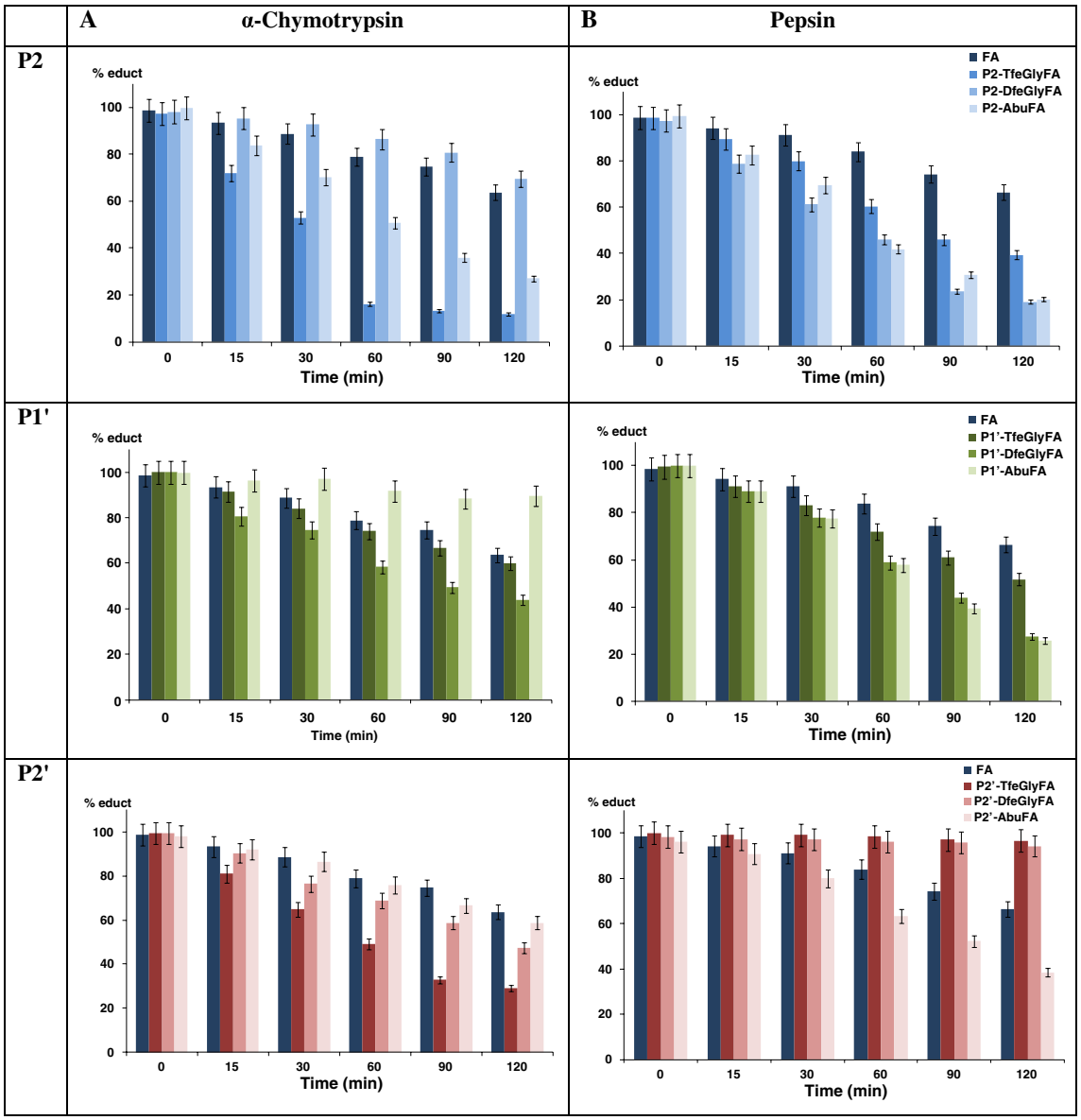


Fig. 3 Time course of changes in the area of the HPLC peaks representing the fluorescent fragments present after hydrolysis of the P2FA, P1′FA and P2′FA peptides by α-chymotrypsin (10 mM phosphate buffer, pH 7.4) and pepsin (10 mM acetate buffer, pH 4.0) at 30 °C

Table 1 Common intermolecular interactions for all valid docking poses into the α-chymotrypsin (Chymo) and pepsin binding sites

Interaction	Ligand		Protein		
	Residue	Atoms	Enzyme	Residues	Atoms
H-bond	P2	Backbone NH	Chymo	Ser214	Backbone CO
H-bond	P1	Backbone CO	Chymo	Gly193, Ser195	Backbone NH's
Hydrophobic	P1	Phe aromatic ring	Chymo	Met192,Cys191-220, Val213	Side chains
H-bond	P2′	Backbone NH	Chymo	Phe41	Backbone CO
H-bond	P2	Backbone CO	Pepsin	Thr77	Backbone NH
H-bond	P1	Backbone NH	Pepsin	Gly217	Backbone CO
Hydrophobic	P1	Phe aromatic ring	Pepsin	Ile120, Phe117, Val30	Side chains
H-bond	P1	Backbone CO	Pepsin	Gly76	Backbone NH
H-bond	P2′	Backbone NH	Pepsin	Gly34	Backbone CO

Position P2

The S2 subsite of α-chymotrypsin forms a shallow hydrophobic groove and generally prefers to accommodate hydrophobic residues (Brady and Abeles 1990). The data obtained from the proteolysis assay suggest that P2-TfeGlyFA is well accommodated by the active α-chymotrypsin and clearly demonstrate that this peptide is the most rapidly degraded of all studied P2 variants (88 % of the initial concentration hydrolyzed after 2 h vs. 36 % for FA and 73 % for P2-AbuFA). Because of the very rapid rate of degradation observed in this case, we selected the P2-TfeGlyFA peptide, and FA as a control, for determination of the Michaelis–Menten parameters. We observed that the Michaelis constant (KM) of P2-TfeGlyFA for α-chymotrypsin is reproducibly lower than that of FA and the rate of degradation (reaction velocity, Vmax) of P2-TfeGlyFA is significantly faster than that of FA (Fig. S2 and Table, Supporting Information). Molecular docking shows that the residue in the P2 position is likely to face the Ile99 side chain of the S2 pocket (Fig. 4a), creating an environment that is well suited to accommodate the trifluoroalkyl side chain.

Interestingly, the presence of a difluoromethyl group in the P2 position significantly reduces the rate of hydrolysis, 30 % of the initial concentration of P2-DfeGlyFA hydrolyzed after 2 h, compared to its natural analogue. This observation might be due to the polarized γC-H methine moiety of the difluoromethyl group (Salwiczek et al. 2009a, b). Nevertheless, the difference in proteolytic stability between difluoro and trifluoro analogues still remains unclear. To summarize the results for the series in which the P2 position was substituted, the following trend was observed from the most stable to the least stable: P2-DfeGlyFA > FA >> P2-AbuFA > P2-TfeGlyFA (Fig. 3).

Position P1′

The S1′ subsite of α-chymotrypsin generally accommodates basic residues with long side chains, due to aspartates 35 and 64 (Schellenberger and Jakubke 1986; Schellenberger et al. 1994). The peptides designed for this study have amino acids with shorter side chains in the P1′ position and molecular modeling shows that the geometric constraints are such that Asp35 and Asp64 cannot be reached when each residue of the substrate is bound to its corresponding subpocket. The docking poses highlight that residues His57 and Phe41, brought into proximity by the disulfide bridge Cys42-58, delineate the edge of the S1′ cavity. The experiments show that the incorporation of Abu disfavors the hydrolysis of the substrate. On the other hand, the two peptides carrying fluorinated amino acids are well accommodated and faster degraded compared to both the Abu variant and the parent FA sequence. This could be due to the fluorine-induced polarity of the side chains (Jäckel et al. 2004, 2006; Salwiczek et al. 2009a, b). Interestingly, the slightly higher affinity of DfeGly for the S1′ subsite could be explained by its ability to create a hydrogen bond (H-bond) between the difluoromethyl group of the substrate (H-bond donor) and the Cys42-58 disulfide bridge (H-bond acceptor). Even though this H-bond is expected to be weak, this might explain the difference between the affinities of P1′-DfeGlyFA and P1′-TfeGlyFA towards α-chymotrypsin. Due to the presence of two electron-attracting fluorine atoms on the same carbon, the hydrogen of the S1′ DfeGly unit is a potential H-bond donor (Erickson and McLoughlin 1995), and can interact with the sulfur (Fig. 4b) (Zhou et al. 2009). The replacement of this hydrogen by a third fluorine atom in the P1′-TfeGlyFA variant apparently decreases its affinity for α-chymotrypsin. This analysis could thus explain the measured trend in peptide stability at the P1′ position (from the most stable to the least stable): P1′-AbuFA > FA > P1′-TfeGlyFA > P1′-DfeGlyFA (Fig. 3).

Position P2′

The S2′ subsite of α-chymotrypsin prefers hydrophobic residues (Antal et al. 2001). Phe39 and Thr151 are the main residues that impart hydrophobic character to the S2′ pocket. We therefore assume that substrates with hydrophobic residues in the P2′ position will display better packing and faster hydrolysis, as illustrated for P2′-TfeGlyFA in Fig. 4c. Accordingly, the observed proteolytic stability decreased together with the hydrophilicity (Salwiczek et al. 2009a, b) of the P2′ residue (Fig. 3): FA > P2′-AbuFA > P2′-DfeGlyFA > P2′-TfeGlyFA (from the most stable to the least stable).

Pepsin

Within the enzyme pocket of this protease, the water molecule stabilized between Asp32 and Asp215 plays a key role in the hydrolysis mechanism (Cornish-Bowden and Knowles 1969; Suguna et al. 1987). Therefore, this water molecule was manually merged into the 3D structure of pepsin before performing the docking study. When a peptide conformation fulfilled the interaction requirements reported in Table 1, then the water molecule was positioned directly under the amide bond between positions P1 and P1′. Similarly, positions P2–P2′ of this peptide were well accommodated by their corresponding enzyme subsites S2–S2′. Therefore, such a conformation was considered to be highly plausible and subjected to more detailed structural investigation.

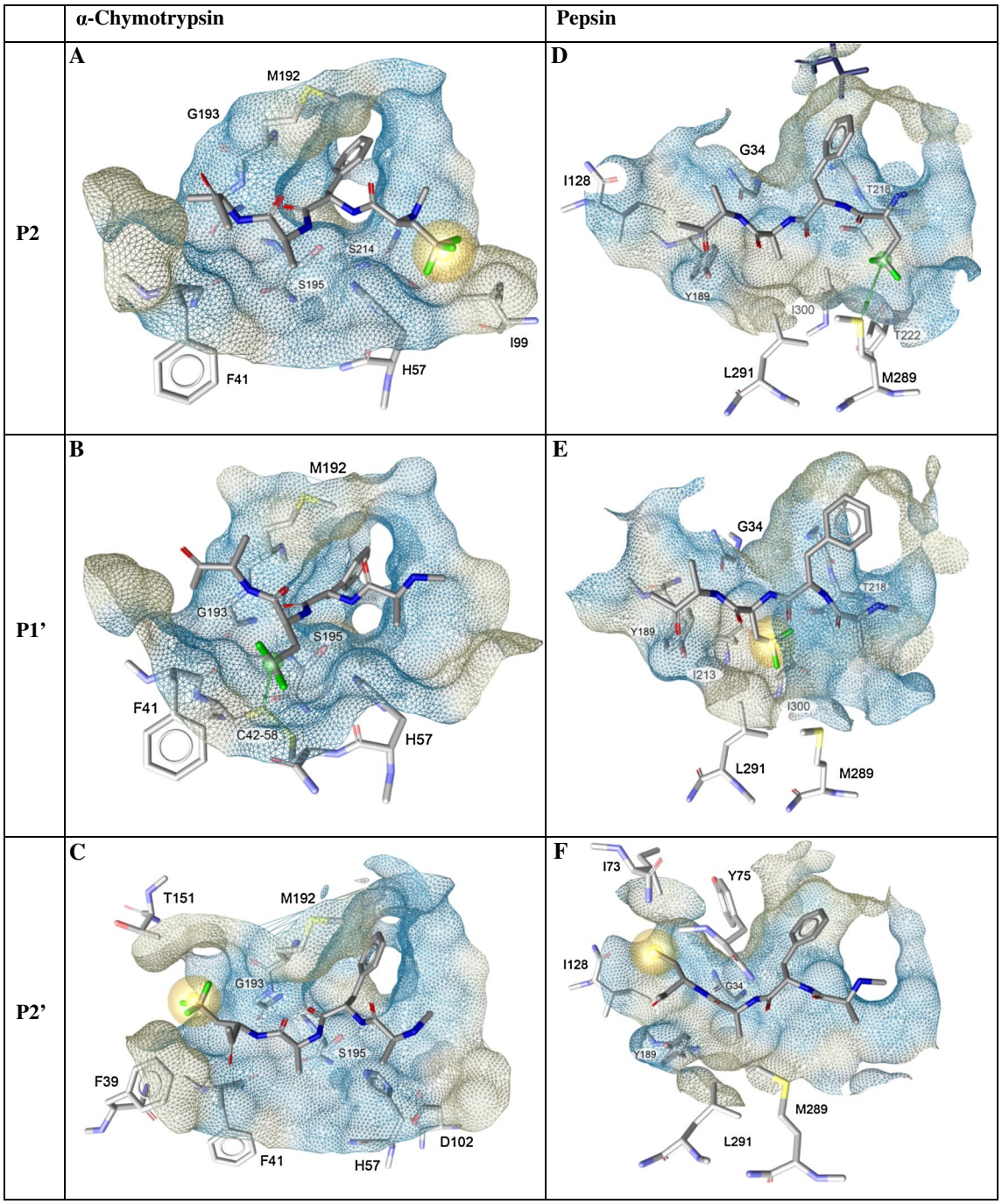


Fig. 4 Suggested binding poses for the FA variants **a** P2-TfeGlyFA, **b** P1′-DfeGlyFA, and **c** P2′-TfeGlyFA bound to α-chymotrypsin, and for **d** P2-DfeGlyFA, **e** P1′-DfeGlyFA, and **f** P2′-AbuFA bound to pepsin. The color code of the surface of the cavity is *yellow* for hydrophobic regions and blue for hydrophilic regions. Highlighted hydrophobic moieties are depicted as *yellow spheres* and the hydrogen bond as a *green arrow* (color figure online)

P2 position

Peptides modified at the P2 position are generally degraded more rapidly, indicating a better geometric fit for pepsin compared to the FA peptide (Fig. 3). P2-DfeGlyFA is the fastest hydrolyzed substrate, with only 19 % of the initial amount of full-length peptide remaining in solution after 2 h of reaction time. Interestingly, analysis of the docking results shows that the DfeGly side chain is in the vicinity of the Met289 side chain within the S2 pocket (Fig. 4d). It

must of course be noted that the side chain of methionine is flexible and should be considered as such in the context of a potential interaction with a ligand.

The observed distance between the sulfur of Met289 and the DfeGly γ-carbon is in the range of 4.5 Å, which could indicate the existence of a weak hydrogen bond, as depicted in Fig. 4d. Behaviors of this kind have been reported, that is methionine acting as H-bond acceptor and a difluoromethyl group of a small molecule acting as H-bond donor (Erickson and McLoughlin 1995; Zhou et al. 2009). Since this polarized, electron-deprived hydrogen is absent in the structure of P2-TfeGlyFA, the trifluorinated variant could still be accommodated but not equally enthalpically stabilized within the enzyme cavity. Finally, the P2-AbuFA variant was also very well accommodated within the hydrophobic S2 subpocket. The extent of proteolytic stability observed is as follows (from the most stable to the least stable): FA > P2-TfeGlyFA > P2-DfeGlyFA = P2-AbuFA (Fig. 3).

P1′ position

Pepsin hydrolyzes peptides that have hydrophobic residues in the P1′ position (Kageyama 2002; Dunn 2002). This specificity is due to the nature of the S1′ subsite constituents, namely Ile213, Ile 300, Leu291 and Thr218. Among all peptides evaluated, the P1′-AbuFA variant is degraded most rapidly (26 % of the initial concentration remaining after 2 h). Peptides containing fluorine in the P1′ positions are also hydrolyzed faster than the FA by pepsin. The DfeGly variant displays a similar rate of reaction, 28 % of full-length peptide (substrate) remaining after 2 h, to the one measured for Abu. The trifluoroalkyl variant is not well degraded as the Abu or DfeGly, thus in this case proteolytic stability phenomenologically corresponds to the steric bulk of the side chain of the nonproteinogenic amino acid (Fig. 3): FA > P1′-TfeGlyFA >> P1′-DfeGlyFA ≥ P1′-AbuFA (from the most stable to the least stable).

P2′ position

The S2′ subsite of pepsin preferentially binds hydrophobic amino acids (Dunn et al. 1986, 1987; Dunn and Hung 2000). Accordingly, the substrate conformations highlighted by the molecular modeling study demonstrate that the P2′ residue interacts with the hydrophobic environment of the S2′ enzyme subsite. In these docking poses, the amino acids of the enzyme involved in interactions with the P2′ amino acid of the substrate are Ile128, Ile73, Ser35, and Tyr75. The experimental data indicate that the introduction of fluorinated amino acids into the P2′ position of the peptide sequence remarkably improves its proteolytic stability, providing evidence that TfeGly and DfeGly are geometrically not well accommodated within the hydrophobic S2′ subpocket. This result can be correlated with the increase in size of the fluorinated variants. In agreement with this interpretation, the peptide variant P2′-AbuFA, smaller than the fluorinated variants, is rapidly hydrolyzed (61.5 %). Furthermore, Abu, more hydrophobic than alanine, may engage in optimal interactions with the hydrophobic S2′ subsite of pepsin as shown in Fig. 4f (Samsonov et al. 2009). Resistance toward proteolysis is thus greater in the case of the fluorinated variants, and can be summarized from the most stable to the least stable as follows: P2′-TfeGlyFA > P2′-DfeGlyFA > FA > P2′-AbuFA (Fig. 3).

Conclusion

In this study, the systematic characterization of nine model protease substrates elucidates the impact of side-chain fluorination on proteolytic stability. Our experiments demonstrate that the incorporation of unnatural building blocks bearing fluoroalkyl side chains into a substrate can significantly affect its proteolytic stability in various ways. We show that the fluorinated variants are generally well processed by the proteases α-chymotrypsin and pepsin, in certain cases even more rapidly than the natural control peptide. This observation indicates that these peptide substrates are geometrically well accommodated within the enzyme subsites S2 to S2′. Molecular modeling was used to identify potential noncovalent interactions between the enzymes and their substrates, in order to rationalize the experimentally determined proteolytic stabilities.

In two particular cases, the observed rate of hydrolysis is significantly reduced upon incorporation of fluorinated residues into the peptide sequence. A strong enhancement in stability towards pepsin is observed when fluorinated amino acids occupy the P2′ position of the FA peptide. This is likely due to the larger solvent accessible surface area of both TfeGly and DfeGly compared to Ala and Abu. In case of α-chymotrypsin, improved proteolytic stability is observed when DfeGly is present in position P2. This can be attributed to the fluorine-induced polarity of the side chain, which appears to disfavor the accommodation of the P2-DfeGlyFA variant within the S2 subsite of the protease.

Our investigations demonstrate that the use of fluorine as a tool for peptide and protein engineering does not automatically improve the stability of peptides towards proteases, as one may expect based on the bioorthogonality of this element. Our findings are in strong contrast to the few proteolysis studies published so far (Meng and Kumar 2007; Meng et al. 2008; Buer and Marsh 2012). However, this contradiction arises mainly because past studies have made use of sterically demanding, extensively fluorinated amino acids. The fluorinated amino acids upon which this

study is based, however, provide a low number of fluorine substituents within shorter side chains. In such cases, fluorine seems often to become the key factor that triggers acceleration of peptide degradation by the here studied enzymes. Thus, our results highlight the fact that fluorine mediates interactions with proteins in ways that are not available to the hydrocarbon analogues. The here reported results are even more important in the field of medicinal chemistry, as this tendency cannot be reconciled with the generally improved metabolic stabilities observed for small drug molecules containing fluorine atoms. Hence, the impact of side-chain fluorination on proteolytic stability of peptides is a complex phenomenon that depends upon the position of the substitution relative to the cleavage site, the type of enzyme, the chemical nature and fluorination state of the side chain, as all of these parameters dictate the precise nature of the noncovalent interactions that the substrate and enzyme will engage in. For this reason, the improved resistances against hydrolysis highlighted with some variants (fluorine at P2′ position with pepsin and DfeGly at P2 with α-chymotrypsin) are valuable observations that have the potential to make an impact on the design of more bioavailable peptides or peptidomimetics. We are currently also investigating other aspartic acid and serine proteases to expand the studies reported here.

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

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