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ORIGINAL ARTICLE

Separate mechanisms for age-related truncation and racemisation of peptide-bound serine

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Abstract Some amino acids are particularly susceptible to degradation in long-lived proteins. Foremost among these are asparagine, aspartic acid and serine. In the case of serine residues, cleavage of the peptide bond on the N-terminal side, as well as racemisation, has been observed. To investigate the role of the hydroxyl group, and whether cleavage and racemisation are linked by a common mechanism, serine peptides with a free hydroxyl group were compared to analogous peptides where the serine hydroxyl group was methylated. Peptide bond cleavage adjacent to serine was increased when the hydroxyl group was present, and this was particularly noticeable when it was present as the hydroxide ion. Adjacent amino acid residues also had a pronounced affect on cleavage at basic pH, with the SerPro motif being especially susceptible to scission. Methylation of the serine hydroxyl group abolished truncation, as did insertion of a bulky amino acid on the N-terminal side of serine. By contrast, racemisation of serine occurred to a similar extent in both *O*-methylated and unmodified peptides. On the basis of these data, it appears that racemisation of Ser, and cleavage adjacent to serine, occur via separate mechanisms. Addition of water across the double bond of dehydroalanine was not detected, suggesting that this mechanism was unlikely to be responsible for conversion of L-serine to D-serine. Abstraction of the alpha proton may account for the majority of racemisation of serine in proteins.

Keywords Serine · Truncation · Racemisation · Aging · Post translational modification · Dehydroalanine

Abbreviations

O-Me	*O*-Methyl ether
Ac	Acetyl
Z	Benzyloxy-carbonyl
DHA	Dehydroalanine

Introduction

Long-lived proteins are found at many sites in the body including the brain (Shapira et al. 1988b; Savas et al. 2012), heart (McCudden and Kraus 2006), lung (Shapiro et al. 1991), connective tissue (Sell and Monnier 2005; Sivan et al. 2008) and lens (Lynnerup et al. 2008). As a result of prolonged exposure to physiological conditions, these proteins undergo numerous age-related changes. It has been proposed that the age-related decline in function of organs, as well as skeletal components, comes about, in part, from the degradation of these long-lived proteins (Truscott 2010). Major modifications include racemisation

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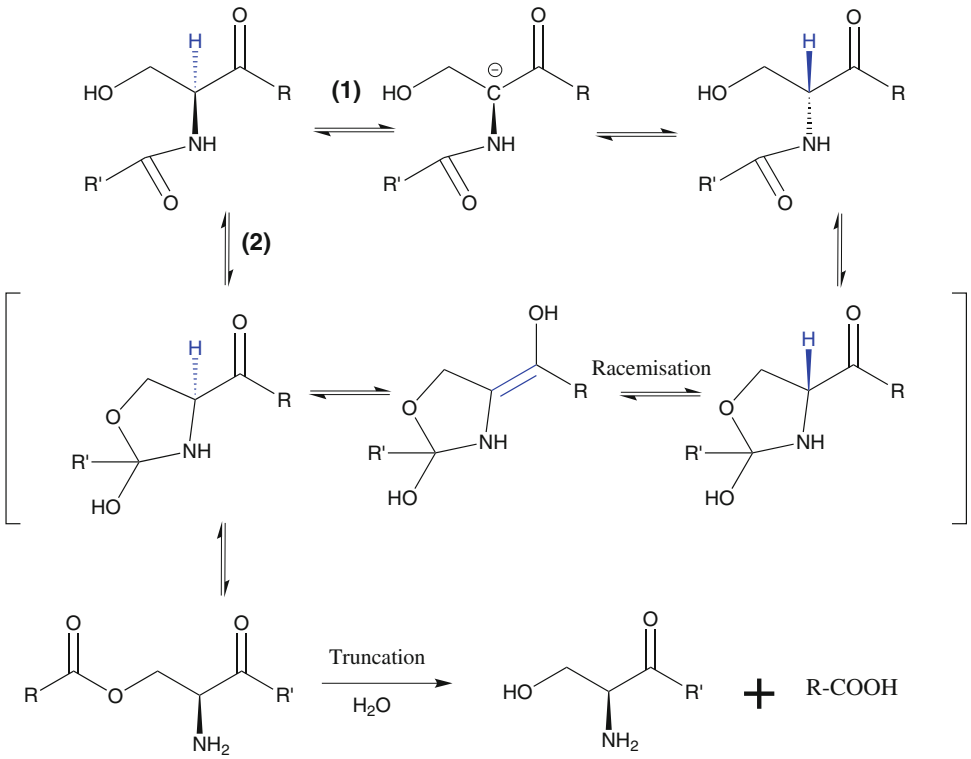

Fig. 1 Two possible mechanisms for racemisation of Ser: (*1*) involving abstraction and re-addition of the alpha proton and (*2*) a cyclisation process. In addition, one route for truncation at the N-terminal side of Ser is shown (*R* and *R*′ represent the rest of the protein)

(Hooi and Truscott 2011; Masters et al. 1977; Fujii et al. 2000), deamidation (Hains and Truscott 2010; Robinson and Robinson 2001; Miesbauer et al. 1994; Groenen et al. 1993; Wilmarth et al. 2006; Lampi et al. 2001), truncation (Takemoto 1995; Srivastava 2000; Srivastava and Srivastava 2003; Harrington et al. 2004; Su et al. 2012) and the covalent addition of reactive metabolites (Argirov et al. 2004; Korlimbinis and Truscott 2006; Larsen et al. 1990; Truscott et al. 2012).

The human lens is an excellent model for studying these processes because it is protein-rich, and contains relatively few major structural proteins, and there is no protein turnover (Lynnerup et al. 2008). In addition, the centre of the adult human lens lacks active enzymes (Dovrat et al. 1984; Zhu et al. 2010) because the proteins in this region were synthesised in utero and have been denatured by exposure to body temperature for decades. Therefore, it is likely that protein modifications that occur with age in the lens centre are non-enzymatic. In the case of the major heat shock protein, α-crystallin, a notable feature of the sequences of two of the most abundant peptides (αA 67–80 and αB 1–18) is that age-related cleavage in the lens occurs on the N-terminal side of Ser residues (Santhoshkumar et al. 2008; Su et al. 2010). Additional Ser truncation sites in older lenses have been observed in αA at Ser 169 as well as between Ser 172 and Ser 173 (Takemoto 1995, 1998; Srivastava and Srivastava 1996).

While truncation of peptides and proteins at the C-terminal side of Asn and the formation of a succinimide ring that leads to racemisation (Clarke 1987) have been well characterised, our understanding of the processes that enable truncation at Ser is incomplete. Ser truncation has previously been demonstrated by incubating peptides, at physiological pH and elevated temperatures (Lyons et al. 2011). A mechanism involving an *N-O*-acyl shift similar to that seen in intein formation (Mills and Paulus 2005), followed by ester hydrolysis was proposed (Fig. 1).

In addition to cleavage at Ser, racemisation of Ser residues is also a common feature of old proteins (Yamasaki et al. 2003; Kubo et al. 2003; Shapira et al. 1988a; Hooi et al. 2012a). With respect to Ser racemisation, a mechanism comparable to Asp/Asn succinimide formation that is observed in long-lived proteins (Geiger and Clarke 1987; Stephenson and Clarke 1989) or in peptides e.g. (Bongers et al. 1992) could be envisaged (Fig. 1).

Although this mechanism is feasible, it is known that racemisation of amino acids can also occur by direct abstraction of the alpha proton (Bada 1972). To determine whether cleavage and racemisation of Ser in peptides are linked as shown in Fig. 1, or occur by separate mechanisms, these processes were examined using unmodified peptides and those where the Ser hydroxyl group was methylated.

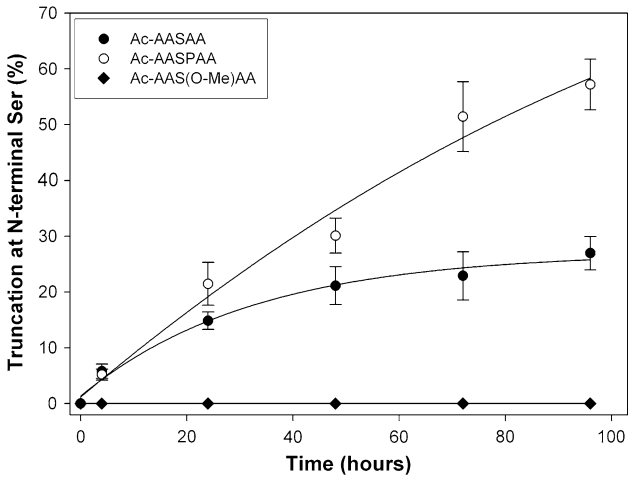

Fig. 2 Truncation at the N-terminal side of Ser as a function of time. Cleavage was calculated based on the amount of Ac-AA formed compared to the starting amount of each peptide. Peptides (Ac-AlaAlaSerAlaAla, Ac-AlaAlaSerProAlaAla and Ac-AlaAlaSer(O-Me)AlaAla) were incubated in sodium borate buffer (100 mM, pH 12.5) at 37 °C

Results

In long-lived proteins, some amino acids appear to be particularly susceptible to age-related degradation. Ser is one example, and it undergoes both racemisation and cleavage. One aim of the current study was to examine these two aspects of Ser modification: racemisation and peptide bond hydrolysis on the N-terminal side of Ser.

Initial experiments were undertaken at high pH, since amino acid racemisation is favoured under basic conditions (Hayashi and Kameda 1980) and at this pH the hydroxyl group of Ser will be partly ionised and therefore more nucleophilic. Some early studies had indicated that exposure of proteins to alkaline conditions could lead to preferential peptide bond hydrolysis next to Ser, Thr and Gly residues (Sine and Hass 1969). Little is known about the details of the cleavage mechanism at high pH or the influence of neighbouring residues.

A series of homologous peptides, Ac-AlaAlaSerAlaAla, Ac-AlaAlaSer(*O*-Me)AlaAla, Ac-AlaAlaSerProAlaAla, Ac-AlaAlaProSerAlaAla and Ac-AlaAlaLeuSerAlaAla were incubated in borate buffer, pH 12.5 at 37 °C for various times and the degree of hydrolysis next to Ser was monitored by HPLC. The results are shown in Fig. 2. Ac-AlaAla was shown to be stable under these conditions.

Cleavage on the N-terminal side of Ser was more rapid in the case of the SerPro sequence than with the Ser Ala peptide, and peptide bond hydrolysis was abolished totally by methylation of the Ser hydroxyl group. These results indicate that the hydroxyl group of Ser is involved in the cleavage mechanism and that the nature of adjacent amino acid residues can significantly affect the extent of the reaction. Additional support for the importance of the amino acids on the N-terminal side of Ser was found when the "reversed sequence", i.e. Ac-AlaAlaProSerAlaAla failed to show any cleavage and Ac-AlaAlaLeuSerAlaAla was also found to be stable under these conditions (Supplementary Fig. 1). As expected, significant racemisation of amino acids was also found. For example, after 6 h, 29.5 % of Ser had been racemised to D-Ser in Ac-AlaAlaSerAlaAla [as judged by co-elution with authentic Ac-AlaAla(D-Ser)AlaAla] and this had increased to 45.5 % by 24 h.

Although these results implicate the hydroxyl group of Ser in promoting hydrolysis of the N-terminal peptide bond, the conditions used are not physiological. These experiments also do not allow an investigation of the mechanism responsible for racemisation of Ser in peptides under biological conditions, since, at basic pH, all amino acids undergo facile loss of the alpha proton (Bada 1972; Smith and Evans 1980). To determine whether the hydroxyl group of Ser plays a role in racemisation, a simpler peptide, Ac-PheSerTyr was employed. The results for incubation of this peptide at pH 7.4, and a homologue where the Ser hydroxyl group was blocked by methylation, are shown in Fig. 2. The identity of each modified peptide was determined using a combination of synthetic standards, ESI MS/MS, chiral HPLC analysis and NMR spectroscopy (data not shown).

Truncation at Ser

Should truncation at the N-terminal side of Ser occur in Ac-PheSerTyr, both Ac-Phe and SerTyr will form. In the case of Ac-PheSer(*O*-Me)Tyr, Ac-Phe and Ser(*O*-Me)Tyr will be generated. To test the stability of the products, separate incubations of Ac-Phe, SerTyr and Ser(*O*-Me)Tyr under the incubation conditions used, revealed that both SerTyr and Ser(*O*-Me)Tyr rapidly degraded via diketopiperazine formation, whereas Ac-Phe was stable. For this reason, the quantity of Ac-Phe produced from each incubation was used to compare the extent of truncation in both tripeptides (Fig. 3).

It is apparent that both Ac-PheSer(*O*-Me)Tyr and Ac-PheSerTyr degraded over time and that both produced Ac-Phe (Fig. 3). The decrease in peak area of the peptide containing the free hydroxyl group was greater and, consistent with this, the generation of Ac-Phe from Ac-PheSerTyr was also larger than the corresponding Ser methyl ether. The data show that Ac-PheSerTyr truncated at approximately double the rate of Ac-PheSer(*O*-Me)Tyr indicating that the presence of a free Ser hydroxyl group increases the rate of peptide bond cleavage.

It was noticeable that the Ser methyl ether also underwent a significant degree of hydrolysis to yield Ac-F. This

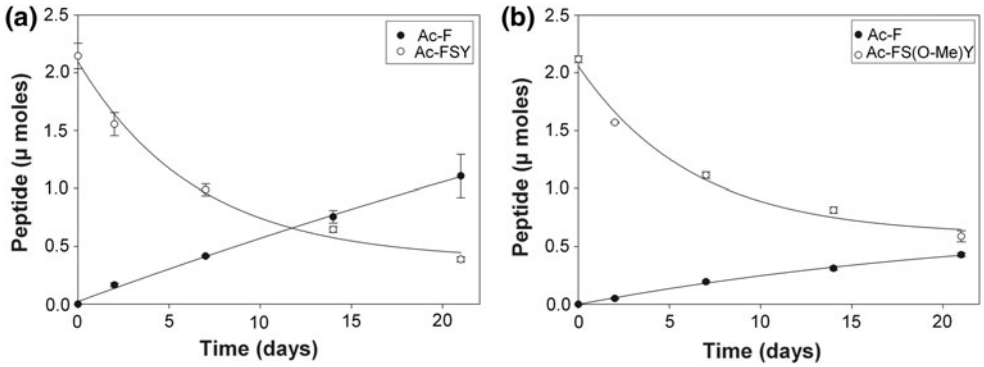

Fig. 3 Time course showing **a** loss of Ac-PheSerTyr and appearance of Ac-Phe, and **b** loss of Ac-PheSer(*O*-Me)Tyr and appearance of Ac-Phe. Peptides were incubated in phosphate buffer (100 mM, pH 7.4) at 85 °C

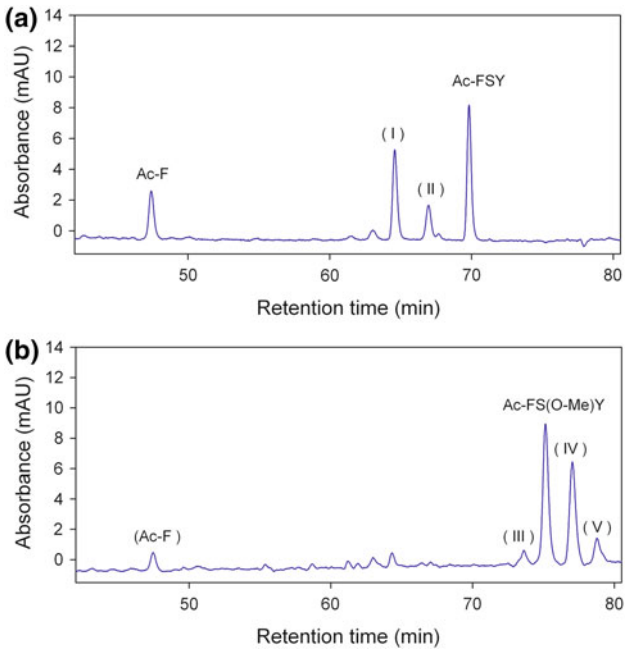

Fig. 4 The effect of methylation of Ser on truncation and racemisation in two peptides: **a** Ac-PheSerTyr and **b** Ac-PheSer(*O*-Me)Tyr. HPLC traces showing the products formed following incubation in phosphate buffer (100 mM, pH 7.4) for 14 days at 85 °C. Detection at 257 nm. **a** (I) Ac-Phe(D-Ser)Tyr and (II) Ac-(D-Phe)SerTyr + Ac-(D-Ser)(D-Phe)Tyr. **b** (III) Ac-PheSer(*O*-Me)(D-Tyr), (IV) Ac-PheSer(D-Ser-*O*-Me)Tyr, and (V) Ac-Phe(D-Ser-*O*-Me)(D-Tyr)

suggests that other factors apart from Ser hydroxyl group-mediated catalysis may be implicated in peptide bond hydrolysis.

Ser racemisation

A major HPLC peak in both the methylated and non-methylated peptide incubations corresponded to the elution time of a peptide where the L-Ser residue had been converted to D-Ser [or L-Ser(O-Me) to D-Ser(*O*-Me)] (Fig. 4). Racemisation of Ser in these peaks was confirmed by a combination of chiral HPLC amino acid analysis and comparison with synthetic peptide standards containing D-Ser or D-Ser(*O*-Me). Significant racemisation of Ser was observed in both incubations. The number of moles of each peptide that underwent Ser racemisation is shown in Fig. 5. The observed plateau in the rate of formation of racemised peptides may be due to peptide degradation via other processes.

Taking into account that the rate of loss of the Ser-containing peptide is greater than that of the methyl ether derivative, the degree to which both peptides racemised was almost identical. This is illustrated by Fig. 6 where the amount of racemised D-Ser is plotted as a function of the amount of peptide remaining at each time point.

Ser as a free amino acid (Bada 1972) and in proteins, (Bruckner et al. 1987) racemises more readily than other amino acids, and it has been suggested that dehydration of peptide-bound Ser may yield dehydroalanine that could potentially form D-Ser following the addition of water across the double bond (Cloos and Jensen 2000). This possibility was investigated in two experimental systems. In one, *Z*-dehydroalanine-*O*-Me was heated (80 °C for 24 h) in a mixture of deuterated DMSO and D_2O (9:1). Analysis by NMR spectroscopy revealed no evidence for addition of water to the double bond. In the other, peptides containing phosphoSer were treated with base to promote β-elimination of the phosphate group (Byford 1991). The dehydroalanine residues that formed were found to be quite stable when incubated at pH 7.4 at elevated temperatures, provided that nucleophiles were blocked or excluded (Wang et al. 2013). These two experiments suggest that if Ser is dehydrated to yield dehydroalanine, it is unlikely that Ser will reform under physiological conditions.

Discussion

Long-lived proteins undergo numerous changes as a result of prolonged exposure to physiological conditions (Masters et al. 1977; Truscott 2010; Ball et al. 2004; D'Angelo et al. 2009). Elevated temperatures have often been used

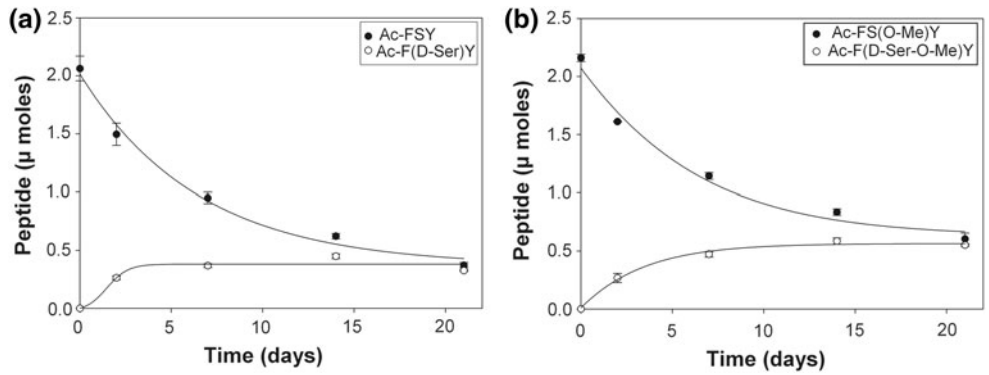

Fig. 5 Effect of methylation of the Ser hydroxyl group on racemisation. Time course showing **a** loss of Ac-PheSerTyr and appearance of Ac-Phe(D-Ser)Tyr, and **b** loss of Ac-PheSer(*O*-Me)Tyr and appearance of Ac-Phe(D-Ser-*O*-Me)Tyr. Ac-Phe(D-Ser)Tyr and Ac-Phe(D-Ser-*O*-Me)Tyr were resolved by HPLC and their structures confirmed by co-elution with commercial standards as well as analysis of L- and D-amino acids by chiral HPLC following hydrolysis in acid. Peptides were incubated in phosphate buffer (100 mM, pH 7.4) at 85 °C

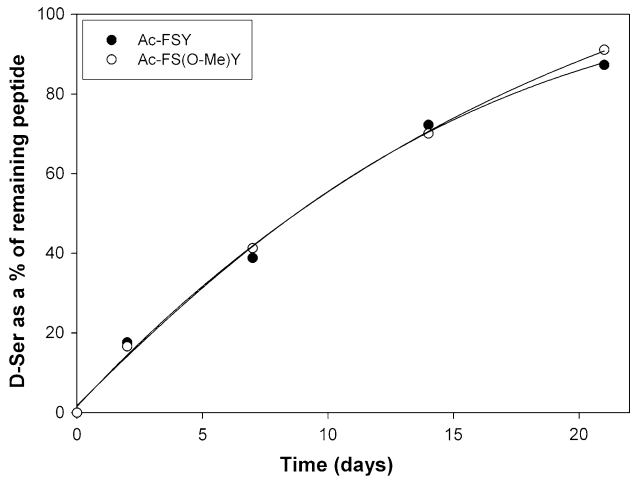

Fig. 6 Ser racemisation in Ac-PheSerTyr and Ac-PheSer(*O*-Me)Tyr. The amount of each peptide which had formed D-Ser, was expressed relative to the amount of Ac-PheSerTyr or Ac-PheSer(*O*-Me)Tyr remaining at each time point. Data were derived from Fig. 5

to simulate the extended periods that these long-lived proteins are exposed to in the body (Alexander and Hughes 1995; Fujii et al. 1996; Cordoba et al. 2005; Liu et al. 2006; Heys et al. 2007; Lyons et al. 2011). In this paper, the role of the Ser hydroxyl group in truncation at the N-terminal side of Ser, and racemisation of L-Ser to D-Ser were investigated. Both racemisation of Ser (Cloos and Jensen 2000; Yamasaki et al. 2003; Hooi and Truscott 2011; Kubo et al. 2003) and truncation adjacent to Ser (Su et al. 2012; Santhoshkumar et al. 2008; Friedrich et al. 2012) have been observed in long-lived proteins, however, the underlying mechanisms remain unclear. If, for example, truncation and racemisation in proteins proceed via a common mechanism, then sites of protein cleavage at Ser would also be expected to show evidence of significant racemisation.

Using simple peptides, we demonstrated that both truncation next to Ser, and racemisation of L-Ser could be detected. The role of the free hydroxyl group was observed most clearly by incubating a series of peptides at alkaline pH (Fig. 4). Under these conditions, some peptides containing Ser were cleaved efficiently in a period of hours at 37 °C. Ac-AASAA was hydrolysed selectively on the N-terminal side of Ser, whereas the corresponding peptide, where the Ser hydroxyl group was methylated, was unaffected (Fig. 4). These data extend some preliminary observations on proteins exposed to pH 12.5 where cleavages next to Ser, Gly and Thr were observed (Sine and Hass 1969).

The nature of the residue adjacent to the Ser was found to have a marked effect on peptide bond cleavage under these conditions. A Pro residue on the C-terminal side of Ser significantly increased the rate of peptide bond cleavage (Fig. 4), whereas insertion of a Pro on the N-terminal side abolished it. Replacement of Ala on the N-terminal side by a more bulky Leu residue also markedly reduced the rate of peptide bond hydrolysis. It is clear that whilst the Ser hydroxyl group is necessary for autolysis, steric factors can significantly affect the time course of the reaction. It is of particular interest that several sites in crystallins where cleavage adjacent to Ser have been detected, involve a SerPro motif (Su et al. 2012; Santhoshkumar et al. 2008).

As expected, it was more difficult to induce Ser racemisation and cleavage at neutral pH and to promote reactions, experiments involved exposure of peptides to elevated temperatures. In most of the experiments reported here, 85 °C was used, with lower rates observed at 60 °C. In cells, protein sequence and tertiary structure, and in particular the proximity of neighbouring functional groups is likely to significantly influence the propensity for reaction of the Ser hydroxyl group. An illustration of

the importance of this feature is the catalytic triad at the active site of serine proteases (Polgar 2005). For example, in the case of trypsin and chymotrypsin, a Ser hydroxyl group in the active site is effectively deprotonated and it then acts as the nucleophile to catalyse peptide bond cleavage of the substrates. Based on our data, a neighbouring group in a cell that facilitates ionisation of the Ser hydroxyl group in a protein may be expected to promote cleavage next to Ser and this will be influenced by the amino acid residues adjoining the Ser. In some cases, cleavage can occur in Ser-containing peptides under quite mild conditions. To illustrate this after 4 weeks in 20 mM phosphate buffer pH 7.4 (containing 10 % D_2O), a sample of PFHSPSY had been completely cleaved into PFH and SPSY (Fig. 7).

The results of this investigation show that a free hydroxyl group increases the rate of truncation at the N-terminus of Ser, but does not appear to influence significantly the rate of Ser racemisation. The *O*-Me derivative cannot form the cyclic tetrahedral intermediate, so if both racemisation were occurring via a mechanism outlined in Fig. 1 then Ac-FS(*O*-Me)Y should have undergone little racemisation. So while a free Ser hydroxyl group may be important for inducing cleavage at the peptide bond on the N-terminal side of Ser, it seems apparent that a different process is responsible for Ser racemisation. Under the conditions outlined here, a mechanism involving an ester-linked intermediate is a likely scenario for truncation.

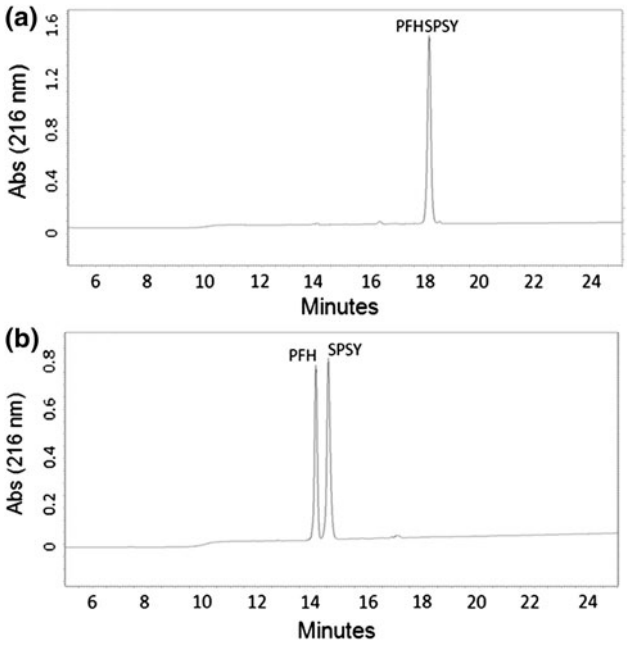

Fig. 7 Spontaneous truncation at Ser in peptides can occur under mild conditions. HPLC profiles of **a** PFHSPSY and **b** after 4 weeks in 20 mM phosphate buffer pH 7.4 containing 10 % D_2O

Racemisation occurs via a different process, with the most likely being direct abstraction of the alpha proton (Bada 1972).

Recently, it has been shown that protein–protein crosslinks occur when phosphoSer residues in long-lived proteins spontaneously decompose via beta elimination to yield dehydroalanine (DHA). These DHA sites are susceptible to attack by amino and sulfhydryl groups (Wang et al. 2013). Therefore, another possible mechanism for Ser racemisation involves dehydration of Ser, to form dehydroalanine (DHA) with subsequent addition of water to the double bond (Cloos and Jensen 2000). In phosphate buffer at pH 7.4, DHA peptides derived by treatment of phosphoSer-containing peptides with base (Byford 1991) were found to be stable in the absence of nucleophiles, such as amino or sulfhydryl groups (Wang et al. 2013). No Ser formation was observed. The stability of a DHA derivative under mixed solvent/aqueous conditions also suggests that a racemisation pathway to D-Ser from L-Ser or phosphoSer that involves a DHA intermediate is unlikely.

In summary, the hydroxyl group of Ser is involved in spontaneous peptide bond cleavage on the N-terminal side of Ser residues in peptides, and therefore presumably long-lived proteins. A mechanism that incorporates an *N*,*O*-acyl shift is the most likely one, as has been postulated for the well-known intein cleavages (Mills and Paulus 2005). Data obtained from peptide incubations at alkaline pH indicated that the nature of the amino acid residues on the N- and C-terminal side of Ser markedly affects the time course of cleavage. In particular, a SerPro sequence appears to be particularly prone to autolysis. In agreement with this observation, sites in long-lived lens crystallins that show a high degree of peptide bond cleavage (e.g. αA crystallin 67–80 and αB crystallin 1–18, Santhoshkumar et al. 2008; Su et al. 2012) occur on the N-terminal side of a SerPro. By contrast, no evidence was obtained to support a role for the hydroxyl group of Ser, or a DHA intermediate, in racemisation. Conversion of L-Ser to D-Ser in old proteins (Hooi et al. 2012a, b; Yamasaki et al. 2003) may best be explained by simple abstraction and re-addition of a proton to the alpha carbon atom.

Materials and methods

Materials

All peptides were synthesised by GL Biochem (Shanghai) Ltd at 95 % purity. Prior to dispatch they were analysed by HPLC and ESI mass spectrometry and shown to be within specification.

Peptide incubations

Incubations were carried out in one of two ways:

(1) In an oven (at 85 °C) using 2 ml glass screw-top vials specially designed to withstand high temperatures. Peptides were incubated in triplicate (1 mg/ml) in 100 mM phosphate buffer adjusted to pH 7.4 at 85 °C. Aliquots (20 µl) were removed and analysed by HPLC.
(2) In an incubator (at 37 °C). Peptides were incubated in triplicate (1 mg/ml) in 100 mM Borate buffer adjusted to pH 12.5 at 37 °C. Aliquots (20 µl) were taken at regular times and analysed by HPLC.

Semi-preparative HPLC purification

A Shimadzu (Nakagyo-ku, Kyoto, Japan) prominence HPLC system controlled by Shimadzu Class VP software equipped with a UV–Vis detector (SPD-20A) and a fraction collector (FRC-10A) was used. Purification of peptides was achieved using a Phenomenex Kinetex (100 mm × 4.6 mm I.D) 2.6 µm 100 Å column at ambient temperature and was monitored at 280 and 216 nm. The gradient was 0 % B (0.1 % TFA) to 60 % B (0.1 % TFA in acetonitrile) over 110 min.

HPLC analysis and quantification

An Agilent (Santa Clara, CA, USA) 1100 HPLC system controlled using Chemstation software and equipped with a PDA detector was used. Incubations were monitored at 280 and 216 nm. Separation of the peptides was achieved using a Jupiter Proteo 4 µm 90 Å column (150 mm × 4.6 mm I.D) at 40 °C. The gradient was 0 % B (0.1 % TFA) to 60 % B (0.1 % TFA in acetonitrile) over 25 min.

Chiral amino acid analysis

Chiral amino acid analysis by HPLC using Marfey's reagent was based on that described by Goodlett et al. (1995). Peptides were hydrolysed in 6 M HCl (110 °C, 6 h) and then lyophilised.

MALDI MS/MS

MALDI-MS analysis was performed using a Shimadzu (Kyoto, Japan) Axima TOF2 mass spectrometer in reflectron positive ion mode. Peptides were prepared in α-cyano-4-hydroxycinnamic acid (8 mg/ml) in 80 % (v/v) acetonitrile, 0.1 % (v/v) TFA.

NMR analysis

Samples were prepared in 50 mm phosphate buffer, pH 7.4 containing 10 % D_2O. Spectra were acquired at 25 °C on an Avance III 800 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a triple-resonance TCI cryoprobe. 1D 1H and 2D 1H-TOCSY experiments were acquired using standard Bruker pulse sequences.

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

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