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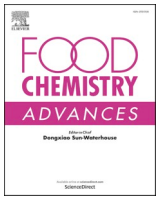

Correlating structure and activity of pepsin enzyme in H_2O and D_2O for the study of gastric digestion

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ABSTRACT

D_2O, an isotope of H_2O, is commonly used as a solvent in neutron scattering; the large difference in scattering length density between H and D can provide better contrast between the sample and the solvent. However, this is of concern for studies using enzymes as the use of D_2O can influence protein interactions (due to differences in hydrogen bonding) and is therefore expected to affect the function, activity and solubility of enzymes. Neutron-based *in vitro* digestion assays on proteins, including those found in food or as oral protein and peptide drugs, often involve different solvents or pH conditions where the activity of the digestive enzyme may not be optimal. Herein, we investigate the structure and activity of the main gastric protease, porcine pepsin, in both H_2O and D_2O at pH values in the range 1 – 8. We showed that the activity of pepsin was lower in D_2O, although the relative change in activity with pH was similar for both solvents. We demonstrated using a combination of SAXS and CD that this relative change in activity was not related to any structural change within the protein but was, rather, linked to relative changes in solubility of the protein.

Introduction

Gastric digestion determines the rate of food breakdown and the emptying of food from the stomach, controlling the delivery rate of nutrients (Kong & Singh, 2008; Le Feunteun et al., 2014). The breakdown of food in the gastric compartment occurs through mechanical shear, enzymatic degradation, and acid hydrolysis. This can be influenced by several environmental factors such as mechanical and hydrodynamic forces, ionic strength, buffering capacity, pH, and enzymatic activity (Kong & Singh, 2008). The gastric aspartic protease pepsin (EC 3.4.23.1, molecular weight 34.55 kDa) is the main gastric enzyme found in the mammalian stomach and catalyzes partial hydrolysis of proteins. It plays a central role in food breakdown and nutrient release, and hence plays a critical role in the accurate assessment of protein digestibility during *in vitro* digestion studies (Brodkorb et al., 2019). Similarly, understanding the influence of pepsin plays a crucial role in developing oral formulations for protein and peptide-based drugs (J. Wang, Yadav, Smart, Tajiri & Basit, 2015). Changes in the gastrointestinal environment such as pH, solvent, ionic strength, or temperature can induce protein conformational changes that may affect the enzyme function. Environment-induced conformational changes in the enzyme structure will affect the digestive enzyme activity and must be accounted for to improve the accuracy of *in vitro* digestion assays (Brodkorb et al., 2019).

Pepsin has two homologous domains, a flexible N-terminal domain (residues 1–172) and a highly folded C-terminal domain (residues 173–326), and consists almost entirely of β-sheets (Sielecki, Fedorov, Boodhoo, Andreeva & James, 1990). Pepsin, like other aspartic proteases, needs to be monoprotonated to be active (Hofer, Kraml, Kahler, Kamenik & Liedl, 2020) and the catalytic site of pepsin is formed by two aspartate residues, Asp 32 and Asp 215 (Dunn, 2002). The environment pH influences the protonation site of these residues; under pH conditions relevant to the stomach environment one catalytic site is protonated and the other deprotonated/charged acting as an acid-base pair (Dunn, 2002).

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The effect of pH on the conformation of pepsin in H_2O has been well characterized for pepsin obtained from several mammalian sources, though porcine pepsin is most commonly used in the literature. In H_2O, porcine pepsin is noted to be active at pH values ranging from 1 to 6 (Davies, 1990; Fruton, 1970; Hofer et al., 2020), with the highest activity occurring at pH 2 (Piper & Fenton, 1965), enabling pepsin to operate in the acidic environment of the stomach. Moreover, porcine pepsin has been shown to retain its conformation in H_2O at acidic pH, transitioning to a denatured state between pH 6 and 7 (Abad-Zapatero, Rydel & Erickson, 1990; Campos & Sancho, 2003; Favilla, Parisoli & Mazzini, 1997; Kamatari, Dobson & Konno, 2003; Lin et al., 1992; Wang & Edelman, 1971). Specifically, an extended conformation occurs above pH 7 due to denaturation, albeit with retention of a reduced secondary structure (Jin et al., 2008; Konno et al., 2000; McPhie, 1989). An intermediate conformation occurs around pH 6.8, and a native/active conformation occurs between pH 1–6 (Campos & Sancho, 2003; Rho, Kim, Min & Jin, 2019). At a pH value of 8, pepsin is irreversibly denatured and inactivated (Jin et al., 2008; Piper & Fenton, 1965). The INFOGEST static digestion protocol, globally used by researchers as a standardized protocol for *in vitro* digestion, uses pepsin dissolved in H_2O at a pH value of 3 to mimic the average stomach environment, maintaining the active and native conformation.

While the influence of pH on pepsin conformation and activity is well understood in H_2O, this effect is poorly understood in other solvents that are becoming more prevalent in the experimental design for *in vitro* digestion studies. Advancing our understanding of structure-activity relationships for pepsin in alternative solvents is critical to ensure neutron scattering data is properly interpreted and correlated with what would be occurring in-body under physiological conditions as digestion proceeds. To date, only a few studies have investigated the effect of alternative solvents or denaturing agents, such as ethanol, 1,4-dioxane, and acetonitrile, on the activity and conformational stability of pepsin (Simon et al., 2007; Simon et al., 1998). Studies examining the effect of solvents or denaturing agents on the structure and activity of pepsin demonstrate that an increase in the concentration of organic solvents or denaturing agents could lead to denaturation, a decrease in the activity of porcine pepsin, and/or changes to the stability and conformation of the protein (Konno et al., 2000; Lin et al., 1993; Rho et al., 2019; Simon et al., 2007).

In this study, we have investigated the effect of D_2O on the structure and function of pepsin as a function of pH. This is important new research as D_2O is the most common solvent used for neutron scattering, NMR, and FTIR experiments, and can offer significant advantages as an alternative solvent. For example, the use of D_2O is crucial to understand the importance of hydrogen bonding in proteins (Sheu, Schlag, Selzle & Yang, 2008), and to identify the primary factors controlling protein folding and stability (Efimova, Haemers, Wierczinski, Norde & Well, 2007; Stadmiller & Pielak, 2018) that may govern protein devolution kinetics under gastric digestive conditions. Moreover, in contrast matched neutron scattering experiments on proteins, D_2O is typically used in combination with H_2O to change the scattering length density of the solvent (Jacrot, 1976; Mahieu et al., 2020). However, recent studies in our laboratory have shown that the use of D_2O as a solvent decreases pepsin activity while influencing the casein gel microstructure (Bayrak et al., 2021). The bulk solvent surrounding the enzyme can influence the mode of action of the enzyme due to thermodynamic variations in the protein hydration shell (Castillo et al., 2005; Castro & Knubovets, 2003). Changes to the solvent isotope, such as replacement of H_2O by D_2O, can also impact enzyme stability and activity via the displacement of water molecules surrounding the enzyme, distorting protein-solvent interactions. It is therefore necessary to determine the enzymatic activity of pepsin in D_2O to ensure that correct enzyme concentrations are selected for *in vitro* digestion experiments involving D_2O. In our previous work, a significant reduction in pepsin enzymatic activity was reported in D_2O compared to H_2O, reducing from ~2700 U mg^{-1} in H_2O to ~750 U mg^{-1} in D_2O (Bayrak et al., 2021). The activity of other enzymes has also been shown to decrease in D_2O compared to H_2O believed to be due to the greater viscosity (Karsten, Lai & Cook, 1995) and lower solubility of D_2O (Broutin, Ries-Kautt & Ducruix, 1995), however the underlying cause for this reduction in pepsin activity remains unknown.

Whilst changes to pepsin structure in H_2O have been reported with changing pH (Jin et al., 2008), the secondary and tertiary conformational changes of porcine pepsin in D_2O have not been characterized to date. This study illustrates the role of solvent environment (H_2O and D_2O) and pH on the activity and solubility of pepsin and identifies the underlying structural interactions affecting enzyme activity. The structure of the pepsin enzyme in solution was investigated for the first time by means of circular dichroism (CD) spectroscopy and small-angle X-ray scattering (SAXS) in combination, and the relationship between enzyme activity, its structural characteristics and pH was determined to better understand its functionality in different environments.

Methods

Materials

The following reagents were purchased from Sigma-Aldrich: deuterium oxide (151,882–1KG), pepsin from porcine gastric mucosa (P6887), the hemoglobin from bovine blood (H6525), trichloroacetic acid (T9159), phosphoric acid (438,081), sodium phosphate dibasic (7558), and hydrochloric acid 37 % (258,148).

Preparation of pepsin solutions at different pH values

Pepsin solutions were prepared in 100 % H_2O or 100 % D_2O and the pH of the solvent was adjusted to a set of $pH_{reading}$ values. The change in $pH_{reading}$ between H_2O and D_2O was accounted for during enzyme activity assays. In an H_2O-calibrated pH-meter, the $pH_{reading}$ in the D_2O solution is converted into pD by adding 0.4 ($pD = pH + 0.4$) (Covington, Paabo, Robinson & Bates, 1968; Rubinson, 2017). Certain $pH_{reading}$ values were selected for structural investigation: pH 2 (the most active form of pepsin Piper and Fenton, (1965)); pH 2.6 (pH at which digestion experiments of D_2O are carried out (i.e. pD =3.0) (Bayrak et al., 2021; Brodkorb et al., 2019); pH 3 (pH at which digestion experiments of H_2O are carried out (Brodkorb et al., 2019)); pH 7 (the fully expanded conformation and inactive form of pepsin (Campos and Sancho, 2003; Konno et al., 2000; McPhie, 1989).

For pepsin solutions made with a mixture of H_2O: D_2O, the pH of the solutions was adjusted separately in 100 % solutions and then mixed by volume. For CD assays, pepsin solutions were mixed to create H_2O: D_2O mixtures of 1:0, 3:1, 1:1, 1:3, and 0:1 by volume.

Pepsin activity assay

The porcine pepsin (EC 3.4.23.1) activity was determined using the protocol described in Salelles, Floury and Le Feunteun (2021) in 100 % H_2O and 100 % D_2O. In brief, bovine blood hemoglobin (2 %, (w/v)) was used as a substrate. Pepsin stock solution (0.1 mg mL^{-1}) was diluted in 10 mM HCl to 10–35 μg mL^{-1} and a 100 μL aliquot incubated with 500 μL 2 % (w/v) haemoglobin at 37 °C for 10 min with shaking. The reaction was then stopped by adding 1 mL trichloroacetic acid (TCA, 5 %). As a control, the hemoglobin solution was incubated at 37 °C for 10 min without pepsin. The reaction was stopped with TCA as above and the enzyme solution was added to bring it to the final sample volume. The samples were centrifuged at 6000 g for 30 min (Eppendorf Centrifuge 5702, Hamburg, Germany) and the absorbance (280 nm) of supernatant was measured in a quartz cuvette using a UV–visible spectrometer.

$$Units \Big/ mg = \frac{[\text{A280Test} - \text{A280 Blank}]\text{x } 1000}{(\Delta\text{t x X})}$$

where Δt is the duration of the reaction and X is the concentration of

pepsin in the final reaction mixture [mg mL^{-1}] (Brodkorb et al., 2019). The pepsin activity in H_2O and D_2O was converted to a relative% of maximum, with the activity at $pH_{reading}$ of 2 in H_2O taken as the maximum (100 %).

Pepsin solubility in H_2O and D_2O

A pepsin solution (10 mg mL^{-1}) was prepared in H_2O and D_2O and suspensions were divided into aliquots for pH adjustment. Pepsin solubility in H_2O and D_2O at pH values from 1 to 9, including 2.6, was studied. The pH of protein dispersions was adjusted using 1 M HCl and 1 M NaOH. The pepsin suspension was mixed for 1 h at 250 rpm and pH was re-adjusted prior to centrifugation. Samples were centrifuged at 10,000 g for 15 min at 20 °C. The total nitrogen and soluble nitrogen in the supernatant were determined using the Dumas method (AOAC method 992.15, 2005), and the protein solubility was calculated as:

$$Solubility\ (\%) = \frac{Supernatant\ N\ concentration}{Total\ N\ concentration}\ x\ 100\%$$

The results were expressed as% of the total protein content.

Circular dichroism spectroscopy

The CD spectra were recorded using a JASCO J-1500 spectropolarimeter (Jasco Corporation, Japan), in the 180–260 nm region using a quartz cell with a 0.1 cm path length. Pepsin solutions at a concentration of 0.2 mg mL^{-1} were found to be the optimum concentration for all pH values in H_2O and D_2O (data not shown). For CD measurements, the pH of the protein solutions was adjusted using 0.1 M phosphoric acid and 0.1 M sodium phosphate dibasic. The sample measurements were obtained by averaging 3 scans per sample for two experimental replicates. The temperature of the cell was held at 25 °C. All CD spectra were analysed using the Dicroweb program (http://dichroweb.cryst.bbk.ac.uk) which calculates secondary structure content by comparing calculated structures and experimental data are smoothed using Jasco Spectra Manager software. The CD values are reported as mean residue ellipticity (MRE), [θ], calculated using a mean residue weight of 106 Da.

Collection and processing of SAXS data

Small-angle X-ray (SAXS) measurements were carried out on the SAXS/WAXS beamline at the Australian Synchrotron, ANSTO. Pepsin structure in solution (10 mg mL^{-1}) was characterised by $pH_{reading}$ values of 6, 7 and 8 in H_2O and D_2O. The samples were prepared in 20 mM phosphate buffer to avoid pH drifts, by mixing sodium phosphate dibasic (Na_2HPO_4, 141.96 g mol^{-1}) and monobasic sodium phosphate (NaH_2PO_4, 119.98 g mol^{-1}). After pepsin (10 mg mL^{-1}) was dissolved in the phosphate buffer, it was filtered through a 0.22 μm filter. Since the autolysis of pepsin can occur, fresh pepsin samples were prepared immediately prior to the SAXS experiments. The samples were loaded into a 1 mm path length cell quartz capillaries and held at 37 °C. For SEC-SAXS, a size-exclusion column GE Healthcare® Superdex S200 5 × 150 was used with high-performance liquid chromatography (HPLC, Agilent 1260). 50 μL of the sample was injected per run, with a flow rate of 0.45 mL min^{-1}. The HPLC column was equilibrated with buffer for at least 30 min for the eluent and the run was recorded by UV (wavelengths 280 nm) and SAXS for over 15 min per sample. Frames were collected every 1.05 s for the full column elution.

2D X-ray scattering patterns were recorded on the Pilatus-2 M detector with an incident X-ray of 12 keV energy. Scatterbrain 2.82 was used for SAXS data reduction. The distance distribution function $p(r)$, the maximum diameter (D_{max}), R_g values, and GASBOR models were obtained using the ATSAS package. Open-source PyMOL v.1.8.4.0 (https://pymol.org/) was used to determine *ab initio*, prediction without homolog structure, and protein structure from the processed scattering data. The structure of pepsin in solvent (without buffer) was also previously investigated using synchrotron SAXS, but pepsin did not seem to be stable according to our results, and a relatively quick aggregation was observed for pepsin in H_2O and D_2O, stored at 4 °C (data not shown).

Statistical analysis

A one-way analysis of variance (ANOVA) was carried out to analyze significant differences in the data using RStudio v3 software (RStudio™, Boston, MA, USA) with the "TukeyHSD" function. A p-value < 0.01 was considered to be statistically significant.

Results and discussion

Pepsin activity and solubility

The activity of porcine pepsin was characterized in the pH range of 1.1 to 5.0 in both 100 % H_2O and 100 % D_2O, using bovine blood hemoglobin as a substrate according to the protocol described in Salelles et al. (2021). As the porcine pepsin enzyme is known to exhibit maximum activity at pH 2, all measured activities in this study are normalized to the measured activity of pepsin in H_2O at pH 2 and are provided as a percentage of this value in Table 1. The characteristic bell shape curve for enzyme activity at different pH values is noted for pepsin in both solvents, with maximum activity observed at pH 2 and a reduction in activity to almost zero at pH 5 (Fig. 1a). We note that the activity of pepsin in H_2O is significantly higher than in D_2O. For example, at pH 2, the pepsin activity in D_2O is ~58 % of that in H_2O (Table 1). However, when comparing the relative activity in H_2O and D_2O at different pH, the shape of the graph (and associated relative change in activity) with increased pH is very similar for both solvents (Table 1), indicating that the reduction in enzyme activity in D_2O is consistent across the pH range examined.

The decreased activity of pepsin in D_2O is not unexpected due to the known decrease in the stability of proteins in D_2O (Hummer, Garde, Garcıa & Pratt, 2000) and the increased strength of the hydrophobic interactions (de Gómez-Puyou, Gomez-Puyou & Cerbón, 1978; Henderson & Henderson, 1969). D_2O creates stronger deuterium bonds compared to hydrogen bonds and therefore replacement of H bonds by D bonds in the hydration shell of the enzyme results in stronger, more stable bonds both within the enzyme and between the enzyme and solvent. In addition, the strength of hydrophobic interactions in D_2O is increased (Cioni & Strambini, 2002; de Gómez-Puyou et al., 1978; Efimova et al., 2007; Henderson & Henderson, 1969; Sheu et al., 2008) and the increased strength of the deuterium bonds in D_2O reduces the motion of the protein which lowers the activity (Sheu et al., 2008). We note that the decrease in enzyme activity in D_2O (compared to H_2O) is in agreement with the majority of previous studies (Bender & Hamilton, 1962; de Gómez-Puyou et al., 1978; Henderson & Henderson, 1969; Sheu et al., 2008), although reaction acceleration was observed in a few cases due to the diphasic activity of certain enzymes, where primary

Table 1
Relative pepsin activity (%) in H_2O and D_2O at pH 2, 2.6, and 3. All values were obtained by averaging determinations done in triplicate. Different superscript letters indicate statistically significant differences across the first two columns together considering pH 2 in H_2O as the 100 % activity (1) and the last column alone (2) ($p<0.01$). * Relative% of activity in D_2O at pH2 was considered as a maximum activity, to allow direct comparison of the rate of change for activity with pH to that of H_2O.

	Relative activity (%)		
pH	H_2O	D_2O	D_2O (relative to D_2O *)
2	$100^{a\ (1)}$	$58 \pm 3^{c\ (1)}$	$100^{a\ (2)}$
2.6	$91 \pm 4^{a\ (1)}$	$52 \pm 3^{c\ (1)}$	$90 \pm 5^{a\ (2)}$
3	$69 \pm 5^{b\ (1)}$	$38 \pm 3^{d\ (1)}$	$66 \pm 5^{b\ (2)}$

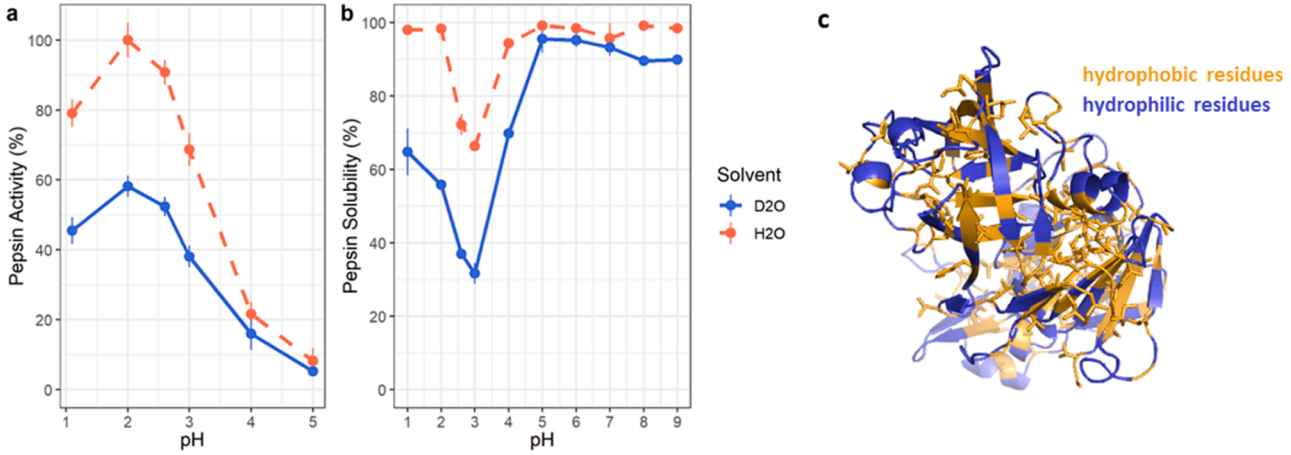

Fig. 1. The pH-dependent activity and solubility of porcine pepsin in H_2O (dashed line) and D_2O (solid line). (a) Pepsin activity for $pH_{reading}$ range of 1 to 5.0. (b) Pepsin solubility for $pH_{reading}$ range of 1 to 9. (c) 3-D ribbon model of the pepsin structure. PyMOL 2.3.4 (Schrödinger) used to visualise protein structure (PDB code: 4PEP). Hydrophobic surface patches are in yellow and hydrophilic surface patches are in blue.

retardation in activity is followed by a secondary acceleration (Macht & Bryan, 1936). While the effect of D_2O on enzyme activity can be temperature dependent (de Gómez-Puyou et al., 1978), in this study the pepsin activity was measured only at 37 °C, of relevance to human digestion under physiological conditions.

All of the activity curves shown here used hemoglobin as the substrate, which is the most common substrate used in enzymatic assays of pepsin activity. We note that the change in activity with pH observed in this study on hemoglobin is slightly less than that observed in previous studies for different pepsin sources; for example, the activity of pepsin at pH 3 was observed to drop by only 30 % in the current work, compared to a drop of 60–70 % by utilizing chicken or ostrich pepsin (Crévieu--Gabriel et al., 1999; Kondjoyan wt al., 2015; Pletschke wt al., 1995). However, we note that these previous studies evaluating pepsin activity as a function of pH employed slightly different methodologies and used different substrates (Salelles et al., 2021) and sources of pepsin such as chicken (Crévieu-Gabriel et al., 1999) or ostrich (Pletschke et al., 1995) all of which can impact the enzymatic reaction rate. Indeed, the pH activity profile of pepsin can vary from substrate to substrate as the optimal pH of proteolysis shifts (Christensen, 1955; Schlamowitz & Peterson, 1959). Taking into account the wide variety of edible proteins, Salelles et al. (2021) showed that pH activity profiles can differ depending on the nature of the protein substrate. For example, substrates, such as wheat gluten or egg white protein, did not produce the bell-shaped curve with maximum activity at pH 2 as observed with hemoglobin (Crévieu-Gabriel et al., 1999; Kondjoyan et al., 2015; Pletschke et al., 1995; Ruan, Chi & Zhang, 2010; Salelles et al., 2021). Considering these differences, the pepsin enzyme activity assay protocol has been optimized and standardized by using the INFOGEST protocol commonly in both industrial and academic research. Hemoglobin is the common substrate used in the standardized protocol, due to its stability, rapid digestion, and limited variation between preparations that causes the digestion rate to vary (Anson & Mirsky, 1932).

In addition to the pH effects, the conformation of the substrate may be affected by the solvent environment, modifying the activity of the enzyme. For many proteases the main driving force behind enzyme-substrate binding is hydrophobic interactions occurring between the side chain of the amino acid substrate and the active site of the enzyme (Fersht, 1999; Klibanov, 2001). The hydrophobic or hydrophilic nature of the solvent can, therefore, influence the exposure of hydrophobic or hydrophilic regions of the substrate, affecting how well the substrate fits into the enzyme's active site and the interactions with its residues. Protein-solvent interactions are one of the most crucial elements stabilising the folded conformation of globular proteins in solution, including hemoglobin which consists of a hydrophobic core at the centre surrounded by polar groups. The protein structure is known to be in R "relaxed" conformation in the presence of oxygen and a T "tense" shape in the absence of oxygen, with the R conformation exposing a greater hydrophobic surface to the solvent. Water molecules, and hence solvent modification, contribute to the T->R conformational transition of hemoglobin. For instance, alcohol-perturbed solvent stabilizes the R conformation of hemoglobin and exposes a greater hydrophobic surface to the solvent (Northrop, 1946). In our scenario, substitution of H_2O with the more hydrophobic D_2O may have hindered the T-to-R transition, decreasing the exposure of hydrophobic regions and altering the pepsin-hemoglobin ligand interactions, which could lead to the reduced activity observed in D_2O compared to H_2O.

In general, the use of D_2O as a solvent increases the hydrophobic effect leading to compact protein structures and decreased flexibility (Efimova et al., 2007; Sasisanker, Oleinikova, Weingärtner, Ravindra & Winter, 2004; Svergun et al., 1998). D_2O is known to stabilize protein structure and reduce solubility (Budayova-Spano, Lafont, Astier, Ebel & Veesler, 2000; Cho et al., 2009; Cioni & Strambini, 2002; de Gómez-Puyou et al., 1978; Efimova et al., 2007; Kresheck, Schneider & Scheraga, 1965; Stadmiller & Pielak, 2018), in particular, the solubility of non-polar amino acids due to its more polar nature (Efimova et al., 2007; Hummer et al., 2000). Pepsin has an average grand average hydropathicity index (GRAVY) of 0.072, which represents the hydrophobicity value for each residue by dividing it by the length of the sequence (Kyte & Doolittle, 1982). The positive GRAVY value indicates the hydrophobic nature of pepsin and suggests that D_2O should be a poorer solvent compared to H_2O due to its more polar nature. The solubility of pepsin was measured in D_2O and H_2O as a function of pH to determine if the observed differences in pepsin activity reflect fundamental changes in solubility. The solubility of all pepsin solutions show a U-shaped curve with the lowest point at $pH_{reading}$ 3 for H_2O and D_2O respectively (Fig. 1b). The shape of the solubility curve with a minimum of pH 3 is consistent for both solvents. This is consistent with the relatively low isoelectric point (pI) of porcine pepsin, which is generally between 2.76 and 2.90 in the literature (Andreeva, Zdanov & Fedorov, 1981; Lee, Choi, Al Adem, Lukman, & Kim, 2020). We note that for pH values higher than 5, the protein solubility is approximately 100 percent for both solvents in reference to the pepsin solubility in H_2O at pH 2.

We found that the solubility of pepsin in D_2O is generally lower than that in H_2O, particularly at lower pH values. This is consistent with the more hydrophobic nature of pepsin, which implies that solubility in H_2O should be greater than that in D_2O (Fig. 1c). In addition, the higher dielectric constant of H_2O (81.5 in H_2O vs 80.7 in D_2O at 273 K) which

governs electrostatic interactions, suggests H_2O would be a better solvent for pepsin than D_2O (Broutin et al., 1995). The reduced solubility of pepsin in D_2O compared to H_2O may also reflect stronger protein-protein interactions in D_2O (as a result of stronger deuterium bonding compared to hydrogen bonding) (Hummer et al., 2000). Proteins in D_2O have a stronger tendency to associate and aggregate which decreases solubility. Data reported herein on the solubility of pepsin are consistent with previous studies which also observed lower solubility in D_2O (Budayova-Spano et al., 2000; Gripon et al., 1997a, 1997b; Sabadini, Cosgrove & do Carmo Egídio, 2006). Additionally, physical constraints imposed by viscosity differences may impact solubility, where the density (1.1 g cm^{-3}) (Nakamura, Tamura & Murakami, 1995) and viscosity (1.2467 mPa at 20 °C) (Horita & Cole, 2004) of D_2O are approximately 10 % and 25 % greater, respectively than H_2O. The higher solvent viscosity of D_2O indicates a greater stiffness of the protein structure due to the nature and strength of the interaction between the solute and solvent (Cioni & Strambini, 2002). The lower pepsin activity in D_2O compared to H_2O is likely to correspond to the lower solubility of pepsin in D_2O compared to H_2O.

Pepsin secondary structure

The far-UV CD spectra of porcine pepsin were measured at $pH_{reading}$ values between 2 and 7 and in a mixed solvent environment of five different H_2O:D_2O ratios (100:0, 75:25, 50:50, 25:50, 0:100). Representative spectra are provided in Fig. 2 as molar residue ellipticity (MRE). The negative band observed at 216 nm is characteristic of the presence of β-sheets in the secondary structure of pepsin as expected (Gore, 2000) and Dichroweb analysis confirmed that the CD spectra are dominated by β-sheets (37 %) similar to previous information on pepsin (44 % β-sheets) (Lee et al., 2020). No significant differences were found in the secondary structure of pepsin over the pH range investigated in H_2O (Supporting Information, Table S1). Moreover, changing the solvent from H_2O to D_2O had no influence on the secondary structure of pepsin at different pH values. This is consistent with previous studies suggesting that the secondary structures of proteins are either unchanged (Dong et al., 1996) or changed only by a modest degree (Dong et al., 1997) following the replacement of H by D. In fact, even with far more disruptive solvents such as ethanol and acetonitrile, no difference in secondary structure was observed with 30–60 % ethanol or 60 % acetonitrile in previous research (Shanmugam, Selvi & Mandal, 2012; Simon et al., 2007). Simon et al. (2007) have reported that porcine pepsin only alters its tertiary structure and results in a functional loss when high concentrations (70 %) of organic solvents are present.

Minor changes in secondary structure were observed at a $pH_{reading}$ of 2 with a slight shift in the negative band at 216 nm to lower wavenumbers observed. This suggests some loss of β-sheet structure, although we note that this is not reflected in a major change to the proportion of β-sheets as calculated by Dichroweb analysis (Table S1). This is consistent with a study by Perlmann and Kerwar (1973) who observed a conformational transition of the polypeptide backbone below $pH_{reading}$ 2. We note that the effect is more prominent in H_2O compared to D_2O (Fig. 2), potentially reflecting the pH difference between D_2O and H_2O ($pH_{reading}$ 2 reflecting pD of 1.6). On the other hand, the effect may be diminished since proteins with predominantly β-sheet type structures are preferentially stabilized in D_2O due to the modulation of hydrophobic interactions and stabilization of deuterium bonds (Cho et al., 2009; de Gómez-Puyou et al., 1978; Efimova et al., 2007; Henderson &

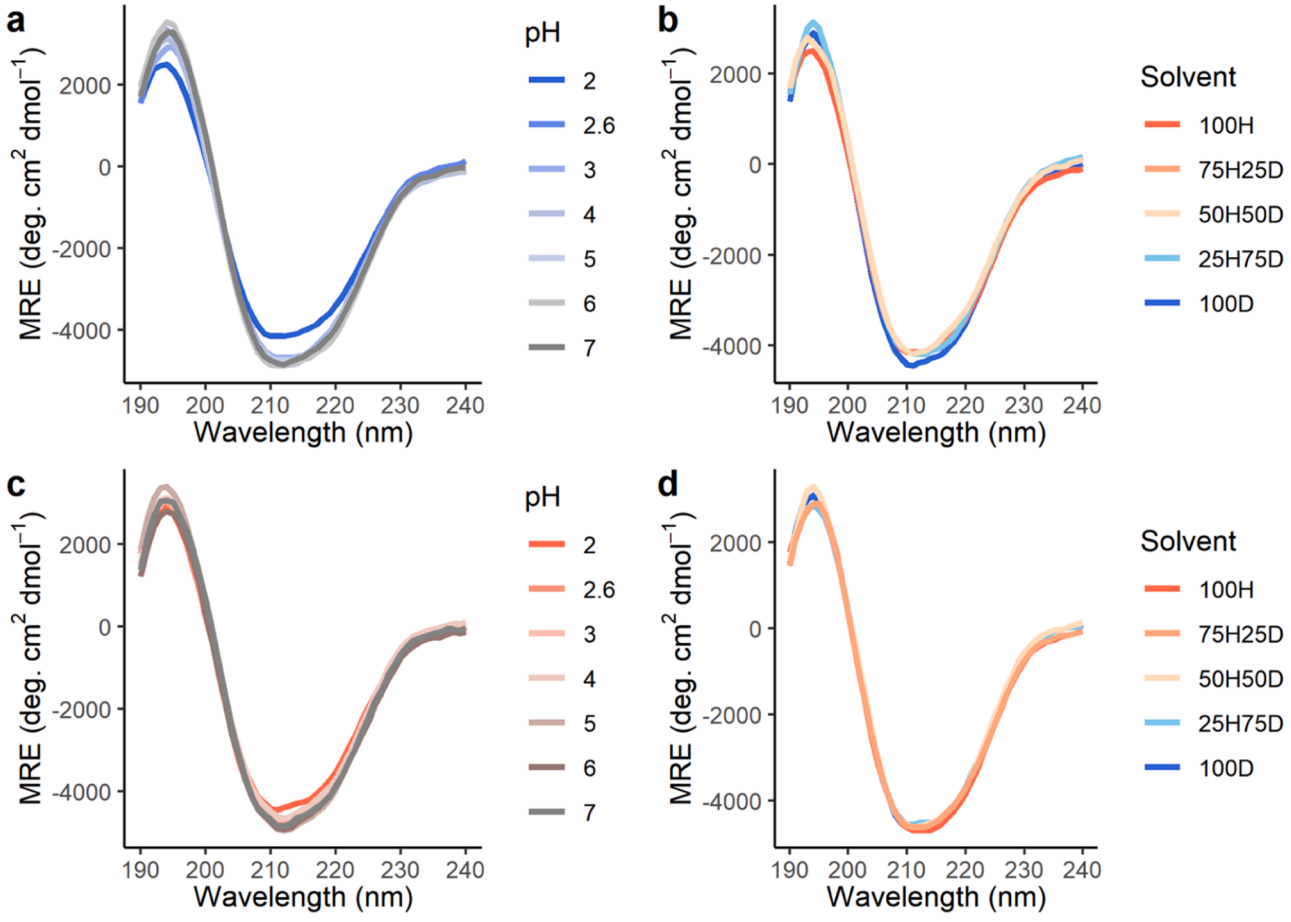

Fig. 2. Far UV Circular Dichroism (CD) spectra of porcine pepsin at $pH_{reading}$ 2–7 in (a) H_2O or (c) D_2O solvent; or in a mixed solvent environment of H_2O: D_2O [100:0, 75:25, 50:50, 25:50, 0:100] at (b) $pH_{reading}$ 2 or (d) $pH_{reading}$ 3.

Henderson, 1969; Sheu et al., 2008). This is similar to the findings of the study of Dong et al. (1997), where the negative band around 216 nm of the CD spectra of thermally denatured pepsin in H_2O is more intense and broadened compared to that in D_2O.

Pepsin structure with SAXS

SAXS was used to evaluate the size, shape and aggregation of pepsin in phosphate buffer at pH values of 6, 7 and 8. These pH values were selected as pepsin is known to change to a denatured state between pH 6 and 7 (Abad-Zapatero et al., 1990; Favilla et al., 1997; Y. Lin et al., 1992) and irreversibly denatures at pH 8 (Jin et al., 2008; Piper & Fenton, 1965). The phosphate buffer selected has a pKa of 7.20 allowing SAXS experiments to be performed at a range of pH values using only one type of buffer, therefore reducing any potential buffer-derived changes to the pepsin structure. Fig. 3a shows the SAXS patterns of pepsin in either D_2O or H_2O buffer solvents at the three pH values. We note that calculations of the radius of gyration (R_g) and Guinier approximation from SAXS data are typically only valid for monodisperse, non-aggregated systems, whereas the SAXS patterns presented here show upturns towards low q consistent with the presence of aggregates. The waiting period for the samples could have contributed to the observed aggregation over time, although it's worth noting that self-digestion effects (autolysis) are improbable due to the low pepsin activity at higher pH values. We have therefore determined pseudo-R_g values from forced linear regression on the Guinier region (arrowed region, Fig. 3a). These pseudo-R_g values can be related to the average diameter of pepsin molecules, and depict the size, compactness and aggregation of proteins (Jin et al., 2008; Rho et al., 2019; Han, Ryan, Rosado, Drummond & Greaves, 2021). Note that for the monomeric pepsin, the pseudo-R_g value is very close to the actual R_g value (~20 Å). A low pseudo-R_g value indicates that the protein is more compact compared to a partially or fully unfolded protein, whereas a larger value can result when aggregation is present. The pair distance distribution function, p(r), has been calculated to represent a real space representation of the distances present in the sample, where a Gaussian curve is characteristic of spheres, such as globular proteins (Fig. 3b). The maximum dimension of the molecule (D_{max}) is additionally estimated from the point where the p(r) function reaches zero (Table S2). Finally, the structural envelope of pepsin is represented by the reconstruction of *ab initio* structural models using the GASBOR program based on the p(r) data (insert, Fig. 3b). We have observed differences in the structural models, and pseudo-R_g and D_{max} values, as a function of pH and solvent.

In H_2O, calculated values of pseudo-R_g and D_{max} were 22.1 Å and 71.0 Å at a $pH_{reading}$ of 6 and 19.9 Å and 63.0 Å at a $pH_{reading}$ of 7. The p(r) curves present symmetrical shapes with a D_{max} of 60–80 Å in both solvents at pH 6 and 7, suggesting monodispersed species present in the systems consistent with a previous study (Rho et al., 2019). Previous studies from Jin et al. (2008) and Konno et al. (2000) showed similar R_g values for porcine pepsin: ~24 Å and ~22 Å for pH 5.6 and 7 respectively. We also found that pseudo-R_g results calculated for pepsin in H_2O

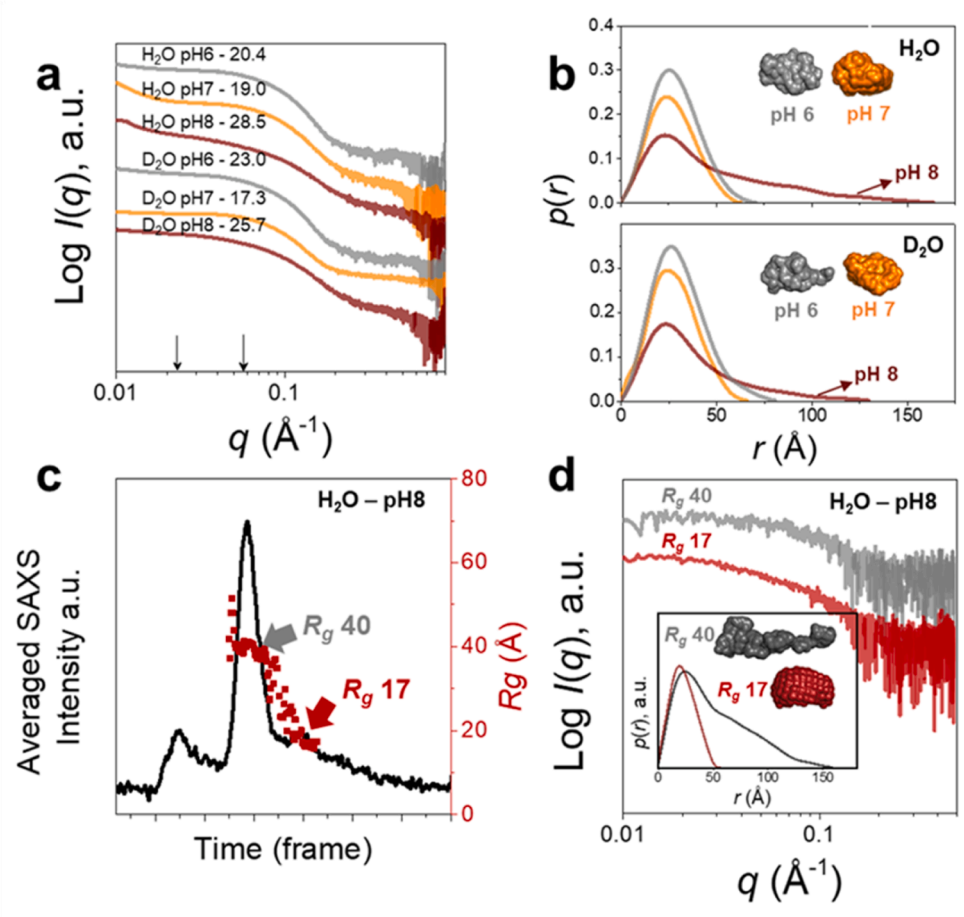

Fig. 3. Estimation of the size, shape and degree of aggregation of pepsin at $pH_{reading}$ of 6 (grey), 7 (orange) and 8 (red) (a) SAXS patterns and the pseudo-Rg values of pepsin in H_2O and D_2O. The arrowed region refers to the Guinier region to calculate the pseudo-Rg values b) Distance distribution function p(r) of pepsin in H_2O (top) and D_2O (bottom) based on the SAXS data using GNOM. The inserts show the corresponding *ab initio* GASBOR model obtained based on p(r) plots. (c) Average intensities of SAXS profiles and the corresponding Rg values through the SEC-SAXS of pepsin as a function of time in H_2O pH 8 (phosphate buffer). The arrowed areas indicate the collected pepsin species with Rg 40 Å (grey) and 17 Å (red) for further analysis. (d) SAXS patterns of pepsin species with Rg 40 Å (grey) and 17 Å (red) collected from SEC-SAXS. The insert shows the p(r)plots and GASBOR models of the two species.

at pH 7 (19.9 Å) are consistent with those reported by Jin et al. (2008) (21.7 Å). Based on the reconstructed *ab initio* structural models using the GASBOR program, the pepsin structure is more compact at $pH_{reading}$ 7 compared to 6 (insert, Fig. 3b).

While pepsin adopts a more compact form in H_2O at a $pH_{reading}$ of 7, a more aggregated and extended form appears at a $pH_{reading}$ of 8 (H_2O pH8, Fig. 3a), in agreement with a previous study (Jin et al., 2008). At pH 8, the pseudo-R_g value significantly increased to 25.6 Å with a D_{max} of 164 (Table S2). At pH 8, the some of the pepsin species can adopt an elongated shape which is discussed later in Fig. 3d, while the $p(r)$ function becomes asymmetric with a peak shift indicating loss of its spherical form by either significant unfolding and/or aggregation (Fig. 3b). Due to the asymmetrical $p(r)$ plots at pH 8 suggesting potential aggregation, size-exclusion chromatography (SEC)-SAXS was carried out to investigate the populations of aggregated and denatured forms of pepsin at pH 8 in H_2O. Fig. 3c shows the average intensity of the SAXS patterns over time of the sample passing through the SEC column for pepsin at pH 8. The overlaid red values provide the R_g value for each SAXS pattern, and the values indicate that protein aggregates and monomers were present in the original sample. The different species with different R_g clearly demonstrate the presence of pepsin aggregates at pH 8 in H_2O. Subsequently, SAXS patterns corresponding to two pepsin species with R_g of ~40 Å and 17 Å were averaged for further analysis. Fig. 3d shows the resulting averaged SAXS patterns corresponding to R_g 40 Å and R_g 17 Å, while the insert shows the $p(r)$ plots and corresponding GASBOR shapes. The R_g 40 Å species of pepsin have an elongated shape with a D_{max} over 150 Å, which is also observed in denatured pepsin with highly concentrated urea present (Rho et al., 2019). However, a compacted form is observed for R_g 17 Å species of pepsin, which is attributed to a monomeric form.

Herein, the structure of pepsin in D_2O was investigated for the first time using synchrotron SAXS. The pseudo-R_g and D_{max} values were found to be smaller than the ones in H_2O (Table S2). Based on the pseudo-R_g, D_{max} and GASBOR model, the pepsin structure is found to be generally more compact in D_2O compared to H_2O (Fig. 3b& Table S2). The more compact structure of pepsin at any pH in D_2O aligns with previous observations of proteins in solvents of different hydrophobicity. On the other hand, when comparing the pepsin structure in D_2O at a pD (pD = pD = $pH_{reading}$ + 0.4) value matching the pH of pepsin in H_2O, one would expect them to be theoretically identical. However, we hypothesise that differences in the actual pH value might indicate variations in the structure. The reduced pseudo-R_g and D_{max} values observed in D_2O suggests less prominent aggregation, where the pD value of 7 corresponds to a $pH_{reading}$ of 6.6. However, the pseudo-R_g was slightly higher in D_2O than H_2O at pH 6 (Fig. 3a), indicating slightly inconsistent aggregation. The aggregation was more pronounced at pH 8 in both D_2O and H_2O, with pseudo-R_g over 25 Å.

Figure S1 shows the Kratky plots of the SAXS data at pH values of 6, 7 and 8 in both H_2O and D_2O. Kratky plots of $q^2I(q)$ vs q, are used to distinguish globular and unfolded conformations of proteins. A bell-shaped curve is present for globular conformations, but on denaturation, the peak becomes weak or disappears with a plateau forming at larger q values. Here, a globular structure is identified by a pronounced peak pattern in the q region below 0.18 $Å^{-1}$. Above 0.18 $Å^{-1}$, the high q Kratky region exhibits linearity with different slopes for each environment, correlated with chain flexibility. The plots with elevated scattering at higher angles suggest that part of the protein is flexible. Pepsin at $pH_{reading}$ 6 shows that pepsin has a globular structure and high chain rigidity for both H_2O and D_2O. At $pH_{reading}$ 7, the overall size of pepsin in D_2O is unchanged but reduced rigidity and greater chain flexibility (as indicated by its asymmetrical peak) were observed suggesting a weak denaturation process (Rho et al., 2019). The denaturation effect was not observed in D_2O at pD 7 but it is expected that the $pH_{reading}$ would remain at 6.6, as a consequence of the 0.4 constant effect between H_2O and D_2O (Covington et al., 1968). A steeper slope with a disrupted peak was observed for pH 8 in H_2O and D_2O, reflecting the greater chain flexibility of pepsin.

Conclusions

This study investigated the effect of the solvent D_2O on the structure, solubility and activity of the pepsin enzyme as a function of pH. The research highlights several novel findings regarding the behaviour of pepsin in different solvent environments and its relationship with pH. Importantly, it demonstrates that the relative solubility of pepsin as a function of pH is similar in both solvents, indicating that the isoelectric point is not correlated with enzyme activity and stability. The study also highlights the different effects of pH and pD (pH adjusted for D_2O) on the protein structure, with the substitution of D_2O with H_2O increasing the compactness of pepsin. Overall, these novel findings enhance our comprehensive understanding of pepsin's structure-function relationship in specific environmental conditions, offering valuable insights for researchers conducting gastric digestion experiments in D_2O environments.

While the activity of pepsin was significantly lower in D_2O, the relative change in activity with pH was similar in both H_2O and D_2O, with the highest activity recorded at pH 2 in both solvents. The solubility of pepsin was also generally lower in D_2O, particularly at lower pH values. However, as for the activity measurements, the relative change in pepsin solubility was similar in both solvents, reaching a minimum at pH 3 (close to the isoelectric point of the enzyme). Therefore, no correlation is found between the activity as a function of pH and the isoelectric point (pI), in agreement with previous analyses of 310 proteins suggesting that the pH-optimum for activity and stability are not correlated with the isoelectric point (Talley & Alexov, 2010). The reduction of enzyme activity and solubility in D_2O (compared to H_2O) likely reflects the increased hydrogen bonding strength in D_2O. We hypothesize that the reduced solubility of the pepsin enzyme in D_2O is also responsible for the lower enzyme activity compared to H_2O.

Analysis of the secondary structure of pepsin via synchrotron SAXS and CD revealed that there is no loss of secondary structure below pH 8 in either H_2O or D_2O, indicating that the change in activity is not correlated with a major change in the secondary structure. The major pH-induced structural changes observed via SAXS measurements to occur at pH 8 are therefore not correlated with changes to the pepsin activity, which has already reached almost zero above pH 5. Analysis of SAXS data with the *p(r)* function and reconstructed models reveal that a transition from the globular pepsin structure to an extended denatured state occurs between pH 7 and pH 8. In contrast to previous studies suggesting that the major structural changes occur above pH 7 due to the irreversible denaturation of pepsin between pH 6 and 7, we observe that pepsin lost its globular form significantly between pH 7 and 8. Although solvent change has a minor effect on the secondary structure at various pH values, the substitution of D_2O with H_2O increases the compactness of the protein as measured by SAXS. The addition of the constant term 0.4 (pD = $pH_{reading}$ + 0.4) when altering pH in D_2O does not produce the same results in H_2O, indicating the different effects of pH and pD on the structure of pepsin.

Overall, our findings demonstrate that the catalytic performance of pepsin is not governed by its structure but depends upon the relative solubility of pepsin in the surrounding environment, as well as the hydrophobicity of the solvent which affects the strength of interactions within the enzyme and its surroundings. Importantly, this study reveals that we cannot assume that the pD-adjusted protein solution in D_2O would have the same effect on the structure of a protein solution in H_2O due to inherent differences in protein solubility and stability. This study improves our comprehensive understanding of the pepsin structure and function under select environmental conditions allowing researchers to effectively conduct gastric digestion experiments for nutrient and drug delivery studies in D_2O environments.

CRediT authorship contribution statement

Meltem Bayrak: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Qi Han:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Tamar L. Greaves:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Susanne Seibt:** Methodology, Investigation. **Haitao Yu:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Juliane Floury:** Conceptualization, Supervision, Methodology, Writing – review & editing. **Jitendra Mata:** Conceptualization, Supervision, Methodology, Writing – review & editing. **Amy Logan:** Conceptualization, Supervision, Methodology, Writing – review & editing. **Charlotte E. Conn:** Conceptualization, Supervision, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The examined data supporting conclusions are presented in the paper. Further data are available upon request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2024.100638.

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