Biophysical Chemistry 172 (2013) 43–52

Contents lists available at SciVerse ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Variant of the *Thermomyces lanuginosus* lipase with improved kinetic stability: A candidate for enzyme replacement therapy

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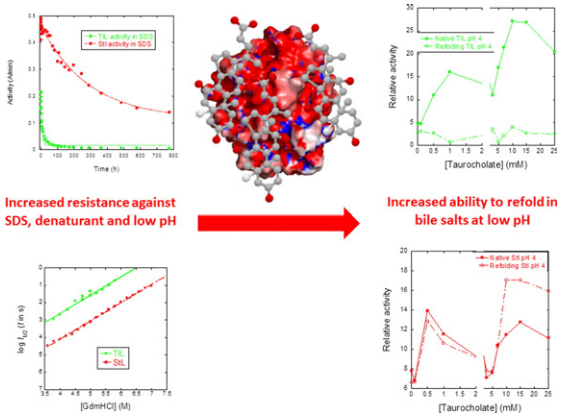
HIGHLIGHTS

- Lipase replacement therapy is hampered by poor stability under gastric conditions.
- A lipase charge mutant is more stable against low pH, denaturants and surfactants.
- The mutant performs better under gastric conditions which include bile salts.
- The mutant shows weak bile contacts and recovers better from low pH than wildtype.
- Increased kinetic stability may help to improve dietary enzyme replacement therapy.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 September 2012
Received in revised form 8 December 2012
Accepted 8 December 2012
Available online 8 January 2013

Keywords:
Refolding
Gastric simulation
Taurocholate
Chemical denaturation
SDS
Acid condition

ABSTRACT

Lipases with high kinetic stability and enzymatic efficiency in the human gastro-intestinal tract may help against exocrine pancreatic insufficiency. Here we mimic gastric conditions to study how bile salts and pH affect the stability and activity of *Thermomyces lanuginosus* lipase (TlL) and its stabler variant StL using spectroscopy, calorimetry and gel electrophoresis. Both enzymes resist trypsin digestion with and without bile salts. Bile salts activate native TlL and StL equally well, bind weakly to denatured TlL and StL at lower pH and precipitate native TlL and StL at pH 4. StL refolds more efficiently than TlL from gastric pH in bile salts, regaining activity when refolding from pH as low as 1.8 and above while TlL cannot go below pH 2.6. StL also unfolds 10–40 fold more slowly in the denaturant guanidinium chloride and the anionic surfactant SDS. We ascribe StL's superior performance to general alterations in its electrostatic potential which makes it more acid-resistant. These superior properties make StL a good candidate for pancreatic enzyme replacement therapy.

Abbreviations: cmc, Critical micelle concentration; DecM, Decyl maltoside; DSC, Differential scanning calorimetry; GC, Sodium glycocholate hydrate; GdmCl, Guanidinium chloride; ITC, Isothermal titration calorimetry; SDS, Sodium dodecyl sulfate; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; StL, TlL mutant; TlL, *Thermomyces lanuginosus* Lipase; TC, Sodium taurocholate hydrate; TFUB, 4-(Trifluoromethyl)umbelliferyl butyrate; TFUO, 4-(Trifluoromethyl) umbelliferyl octanoate.

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http://dx.doi.org/10.1016/j.bpc.2012.12.003

1. Introduction

Chronic pancreatitis, cystic fibrosis and other pancreatic diseases lead to exocrine pancreatic insufficiency and reduced production of fat-digesting lipases. This in turn leads to fat malabsorption, steatorrhea and malnutrition [1,2]. The current prevailing treatment is pancreatic lipase replacement therapy with each meal [3]. A supplemental lipase administered orally to treat pancreatic enzyme insufficiency has to survive passage through the esophagus, stomach and duodenum to work in the small intestine. In healthy people, the average basal pH in the stomach is 2.16 +/− 0.09 in men and 2.79 +/− 0.18 in women [4], though the pH can rise to as much as 6 when food comes into the stomach [5]. Low pH promotes protein denaturation, facilitating proteolytic degradation and further digestion in the small intestine. From the stomach, food is transferred to the duodenum where many digestive enzymes are activated. Here acidic chyme from the stomach is neutralised by pancreatic bicarbonate [6] and 2.5–10 mM bile salts from the liver [7]. Patients with pancreatic insufficiency have bile salt concentrations below 1 mM [8] and >6-fold reduction in bicarbonate [9,10], weakening the ability to neutralise the acidic fluid from stomach and keeping duodenal pH values below pH 4 [9,11]. The normal intestinal pH range is 4–5.5 in the duodenum, 5.5–7 in the jejunum and 7–7.5 in the ileum, colon, and rectum [12,13] though there is high individual variability. The digestive juice from pancreas also contains other concentrated digestive enzymes including trypsin, which further digests the acidic denatured protein from the stomach.

Bile salts emulsify large water-insoluble fat globules into smaller droplets, providing easier fat access for water soluble lipases in the aqueous environment of the intestinal tract. In humans, the salts of taurocholic acid and glycocholic acid represent ~80% of all bile salts [14]. Bile salts are so-called facial amphiphiles comprising a large, rigid, and hydrophobic steroid moiety with hydrophilic groups (typically two or three hydroxyl groups) and amino acids conjugated to the steroid carboxylate group, forming weak acids. As a result of this structure, bile salt micelles consist of a much smaller number of amphiphile molecules than classical surfactants (typically just 2–4 [15] in contrast to ~50–60 for sodium dodecyl sulfate [16]). Hence these micelles are very dynamic and can vary from oblate to spherical and prolate, facilitating formation of dynamic aggregates to solubilise and transport lipid soluble compounds [17].

Effective therapy has been limited by lipases' stability and activity in the gastrointestinal tract [18]. Lipases have to survive potentially inactivating influences such as low pH in the stomach, pH transitions to more neutral conditions, the detergent action of bile salts and digestive proteases in the duodenum. To avoid degradation and to show satisfactory efficacy, porcine pancreatic lipase, the most widely used commercial supplementary lipase, is applied by using enteric coated tablets or pellets in patients with pancreatic enzyme insufficiency. Allergy to porcine proteins and religious concerns with pork products limit its use in some patients [18]. Unfortunately the acid- and protease-stable fungal and mammalian lipases also have disadvantages. *Rhizopus arrhizus* lipase is inhibited by bile salts and rapidly degraded by trypsin. *In vivo* studies also show that the effect of *R. arrhizus* lipase is smaller than a classical pancreatic preparation, and that lipolysis probably occurs in the stomach rather than the intestine [19]. *Aspergillus niger* lipase is ineffective in treating pancreatic steatorrhoea in humans under the conditions of the experiment [20] despite clinical efficacy in dogs with pancreatic steatorrhoea [21]. *Yarrowia lipolytica* lipase is sensitive to interfacial denaturation [22]. A recombinant dog gastric lipase was withdrawn in September 2008 due to its low activity [23] and high sensitivity to trypsin proteolysis [24]. Recombinant human lipases produced in non-eukaryotic cell system lack post-translational modifications, and this may interfere with enzymatic activity [25,26]. There is therefore interest in new enzymes to improve the efficiency of action in the gastrointestinal tract [18].

Kinetically stable proteins are potentially very useful in this context. Such proteins have extremely high activation barrier separating the folded and denatured state, enabling them to retain their native structure and activity under denaturing conditions, even though the overall change in Gibbs free energy (ΔG) favours the unfolded state. The 269-residue *Thermomyces lanoginosus* Lipase (TlL, also known as Lipolase® [27]) is a commercially available enzyme in the detergent industry that catalyses the degradation of triglycerides. TlL shows high kinetic stability against anionic surfactants, enabling it to work under harsh chemical conditions which denature most other proteins. Furthermore, the protein does not require bile salts and colipase for activation [28], making TlL suitable for clinical applications. The kinetic stability of TlL shrinks with decreasing pH in anionic surfactants such as SDS but not in nonionic surfactants such as DecM [29]. TlL and its variants are widely used as commercial detergent enzymes, since they are quite stable and activated by surfactants. Based on this, we reasoned that TlL and variants hereof might be stable and active within the digestive tract. Here we put this hypothesis to the test and explore TlL's potential as a dietary supplement. We present a detailed comparative study of the activity and stability of TlL and its more stable genetically engineered variant StL under simulated gastrointestinal conditions, namely the transfer to acidic to neutral pH in the presence of two representative bile salts. StL shows more than twice the levels of activity recorded for wild type TlL after exposure to simulated gastrointestinal conditions. The mutations also significantly improved the enzyme's stability against unfolding at low pH and in denaturants as well as its kinetic stability in surfactant. These data highlight StL as a potential candidate for the treatment of pancreatic enzyme insufficiency if applied in combination with suitable amylolytic and proteolytic enzyme preparations.

2. Materials and methods

2.1. Chemicals

Sodium acetate was from Merck. All other chemicals, including taurocholate (TC, purity > 97%) and glycocholate (GC, purity > 97%), were from Sigma-Aldrich (St. Louis, MO). All chemicals were of the highest grade available. Experiments were carried out at 37 °C unless otherwise stated.

2.2. TlL and StL preparation

TlL and StL were generously provided by Novozymes A/S as a liquid formulation, diluted with water, dialysed extensively against 1 mM Tris pH 8 at 5 °C for 24 h, centrifuged to remove small amounts of precipitate and stored in aliquots at −80 °C. Protein concentration was determined using a molar extinction coefficient of 37,275 M^{-1} cm^{-1}.

2.3. Activity assays

Activity assays of TlL and StL in the presence of bile salts were performed using a concentration of 6 nM enzymes and 0.2 mM 4-(Trifluoromethyl) umbelliferyl butyrate (TFUB) as described [29] except that enzymes were denatured in 10 mM glycine pH 2.4 in the absence of urea (rather than 6 M urea pH 2.0) to simulate gastric conditions more closely. TFUB is a convenient lipase substrate [28–32] since its short C_4 chain (estimated cmc several M) prevents it from forming micelles on its own or together with other surfactants below the surfactant cmc [28]. The long chain length substrate 4-(Trifluoromethyl) umbelliferyl octanoate (TFUO) showed similar trends, but yielded poorer data quality and suffered from pronounced autohydrolysis at pH values above 8 (data not shown). For experiments on the starting pH effect on recovery of TlL and StL activity and structure at pH 6 in TC, proteins were initially incubated in 10 mM buffer pH 1–5 for 1 h, then incubated in 50 mM MES pH 6

and different concentrations of TC for 5 min. Activity and fluorescence were then measured as described [29]. Activity assays for TlL and StL in SDS at different time points were carried out at 25 °C as described [28].

2.4. SDS-PAGE

SDS-PAGE was performed as described [29] to check the folding state of TlL and Stl after incubation with bile salts at different pH values from the native or pH 2.4-denatured state.

2.5. pH stability of TlL and Stl

Native TlL and StL were incubated at pH 1–8 using 10 mM buffer for 0.5 h and structural changes were monitored in two ways. Firstly by fluorescence (excitation 295 nm, emission 342 nm) on a LS-55 Luminescence spectrometer (Perkin-Elmer Instruments, UK). Secondly, by far-UV circular dichroism based on spectra recorded on a JASCO J-715 spectropolarimeter (Jasco Spectroscopic Co. Ltd., Japan) using a data pitch of 0.2 nm, 50 nm/min scan speed, 2 s response time, 1 nm bandwidth and 3 accumulations. Buffers used were Gly pH 1–3.6, NaOAc pH 3.8–5.4, MES pH 5.6–6.6 and HEPES pH 6.8–8. Native TlL was also incubated at pH 2–8 in 10 mM buffer for 0.5 h to check its folded state at different pH values by SDS-PAGE. Band intensity was subsequently quantitated by scanning densitometry using a conventional scanner in combination with the programme ImageJ.

2.6. Differential scanning calorimetry (DSC)

DSC experiments were carried out using a VP-DSC microcalorimeter (Microcal, USA). Experiments were performed using 0.54 mg/ml TlL, different concentrations of TC or GC, 0 or 0.1 mg/ml thermolysin and a scan rate of 90 °C/h as described [33,34].

2.7. Proteolysis

Protease assays were carried out using a final concentration of 240 μg/ml TlL, StL or casein, 0–200 μg/ml trypsin and 0–15 mM TC or GC in 50 mM buffer pH 6–7–8. At different time points, solutions were taken out and mixed with 2× loading buffer (containing SDS and DTT), boiled for 10 min and run on SDS-PAGE. Buffers used were MES pH 6 and 7 and HEPES pH 8.

2.8. Isothermal titration calorimetry

Calorimetric measurements were conducted on an ITC apparatus (MicroCal Inc., Northampton, MA, USA). The reference cell was filled with water and the sample cell was loaded with a solution of 0.2–4 mg/mL TlL, StL or 1–3 mM TFUB. The cell solution was titrated with aliquots of 5 μl of 100 mM TC, GC or SDS in buffer (10 mM Tris pH 8.0, 50 mM MES pH 6.0 or 50 mM NaOAc pH 4.0). Experiments were done at 25 °C (SDS) or 37 °C (TC and GC). Heat signals were integrated using Origin.

2.9. Pyrene

Pyrene is a highly hydrophobic molecule with low solubility in water (2–3 μM). The ratio of the emission peaks at 372.5 (I_1) and 383.5 nm (I_3) can be used to evaluate changes in the polarity of its environment caused by micelle formation [35]. 0 or 2 μM TlL was mixed with appropriate amounts of TC or GC and buffer. After equilibration for 30 min, pyrene was added from a 50 μM stock in ethanol to a final concentration of 1 μM. Scans were performed from 360 to 410 nm using an excitation wavelength of 335 nm and excitation/emission slits of 5/2.5 nm.

2.10. Unfolding kinetics

Different concentrations of GdmCl in 10 mM buffer or SDS in 50 mM buffer were mixed with 20 μl of 50 μM TlL or StL (final volume 1 ml) in a 1.5 mL cuvette using magnetic stirring. Fluorescence was recorded on a Cary Eclipse fluorescence spectrophotometer (Varian, CA, USA) using excitation at 295 nm and emission at 338 nm. Slit widths of 5 nm were used. The final TlL or StL concentration was 1 μM and the temperature was held at 25 °C unless otherwise stated. Rate constants were obtained by fitting fluorescence data to a single exponential decay.

2.11. Native TlL interaction with bile salts (37 °C) and native StL interaction with SDS (20 °C)

TlL was equilibrated with varying concentrations of bile salts and StL was equilibrated with varying concentrations of SDS in 50 mM buffer at appropriate pH values. Fluorescence and CD spectra were recorded as described above. Temperature scans were measured using 90 °C/h scan speed from 20 to 100 °C.

2.12. Refolding of TlL in bile salts and StL in SDS

25 or 125 μM TlL was initially unfolded for one hr in 10 mM glycine pH 2.4 for bile salts refolding experiments. 25 or 125 μM StL was initially unfolded in 10 mM glycine pH 2 and 6 M urea for SDS refolding experiments. Refolding was initiated by a 25 times dilution with 50 mM buffer at the appropriate pH at 37 °C (TlL) or 20 °C (StL). Folding was monitored over time by fluorescence or CD.

3. Results

3.1. TlL is activated in bile salts at 37 °C over a wide pH range

In order to be effective, digestive supplemental enzymes must be stable and active within the gastrointestinal tract, where they are exposed to various potentially inactivating influences such as low stomach pH, amphiphiles and digestive proteases [36]. We have previously shown that TlL displays excellent activity and stability at high concentrations of anionic surfactant and a range of pH values [28]. In the present study we simulate gastrointestinal conditions as follows: Firstly we incubate TlL at pH 2.4 and 37 °C for 1 h (gastric conditions). Secondly we transfer TlL to pH 4, 6 and 8 in the presence of different concentrations of TC and measure the activity toward the synthetic substrate TFUB at 37 °C. This pH-range corresponds to conditions spanning the duodenum and small intestine; the pH rises and the bile salt concentration decreases as one progresses into the small intestine. As reference, we measured the activity of native TlL (not previously exposed to pH 2.4) in the same conditions. At all pH values, 0–0.5 mM TC enhances the activity of native TlL toward TFUB 2–3 fold (Fig. 1A, B and C). At higher bile salt concentrations, the activity declines to a plateau close to the value measured in the absence of bile salts. This decline is steeper at pH 8 than at pH 6 and pH 4. Comparison of native and refolded TlL shows > 70% activity recovery at pH 8, but at pH 6 activity recovery is only ~ 50% below cmc and declines to zero above cmc. No significant activity is regained with or without bile salts upon attempting refolding at pH 4. A similar survey of the effect of glycocholate on TlL enzymatic activity shows the same steep rise and decline in enzymatic activity (data not shown).

3.2. TlL is stable over wide pH range in bile salts at 37 °C but is trapped in an inactive state even in the absence of bile salts when refolding from low pH to pH 4–6

To correlate these activity effects with structural changes to TlL, we monitored changes in native and refolded TlL's Trp fluorescence

(TF) and far-UV CD spectra (CD) upon incubation or refolding in the presence of bile salts (Fig. S1A–C in Supplementary Information) and CD (Fig. S1D–F). At pH 8, neither native nor refolded TlL are affected by TC in terms of TF and CD and do not aggregate (Fig. S1G). At pH 6, neither TF and CD of native TlL change with increasing TC concentration, but refolded TlL (at a concentration of 5 μM used to record CD spectra) aggregates and can be spun down; this effect is gradually reversed as the bile salt concentration increases to ~10 mM (Fig. S1H). At pH 4, the difference in TF and CD between native and low-pH-treated TlL is even larger and is observed over all TC concentrations (Fig. S1I). These structural changes are further confirmed by SDS-PAGE (Fig. S2A–C). Native TlL at pH 8 forms streaking bands, which we attribute to weak binding to SDS, leading to a mixed population possibly with different degrees of SDS binding [29]. Denatured TlL (10 mM SDS pH 8, boiled) moves as a single band, reflecting a more uniform degree of SDS binding [29]. All samples are mixed with loading buffer containing 2% SDS at pH 8 and loaded without prior boiling; these conditions trap TlL in the state it was in prior to addition of SDS, since native protein resists unfolding in SDS at pH 8 while denatured TlL cannot refold in the presence of SDS [29]. Native TlL maintains the native (streaking) structure in TC and GC at pH 8–6–4. The migration of refolded TlL in TC and GC at pH 8 suggests that it can recover in TC and GC at pH 8 and migrates as the native TlL on SDS-PAGE. In contrast, low-pH-treated TlL migrated differently on SDS-PAGE at pH 6–4, indicating that refolded TlL is trapped in another state at pH 6–4 with or without TC and GC. TlL in this trapped state is in an aggregated state, since it can be pelleted by centrifugation (Fig. S1H–I). However, the aggregate dissolves upon return to pH 8 (data not shown).

In summary, fluorescence, CD spectra and SDS-PAGE show that native TlL is stable over a wide pH range in bile salts at 37 °C, and that TlL can refold at pH 8 but not at pH 6–4.

To test the robustness of TlL against proteolytic degradation during the digestive process, we incubated it with trypsin, the dominant protease in the intestinal tract. However, SDS-PAGE showed no significant degradation by trypsin (Fig. S3A) at pH 6–8, even at high concentrations of bile salts (Fig. S3B). This is not due to inhibition of trypsin activity by bile salts; natively unfolded casein was digested by trypsin in bile salts (data not shown). TlL's resistance to degradation is confirmed by differential scanning calorimetry (Fig. S3C and D) and thermal CD scans (data not shown) at pH 8, which showed no reduction in melting temperature at up to 20 mM TC.

We further investigated the interaction between 27 and 81 μM TlL and bile salts using isothermal titration calorimetry (ITC). However, no signal was detected (data not shown), indicating that the interactions are weak or do not involve significant changes in enthalpy. Further indications of interactions between bile salts and TlL are provided by pyrene fluorescence data. Pyrene fluorescence is sensitive to solvent polarity and can be used to monitor formation of micelles, both those free in solution and formed in the presence of proteins [37,38]. When we titrate pyrene with increasing amounts of glycocholate (Fig. 2A–C), TlL does not affect micelle formation at pH 8 and 6. However, at pH 4, pyrene fluorescence increases more steeply at low bile salts in the presence of TlL. Similar effects are seen for taurocholate (data not shown).

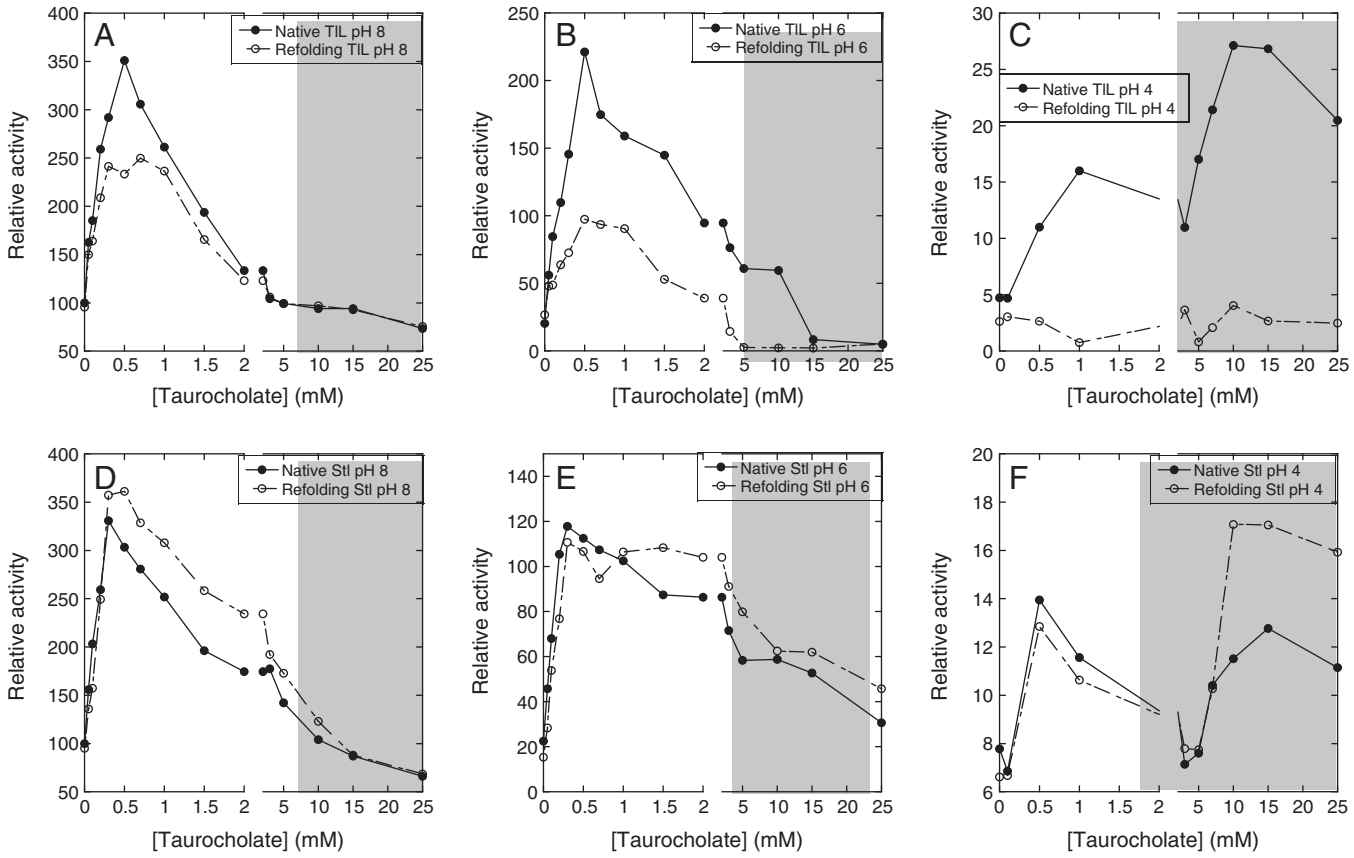

Fig. 1. Relative activity of native and low-pH-treated (refolding) TlL(A–C) or StL (D–F) towards TFUB at pH 4–8 in the presence of taurocholate. Activity units were normalised so TlL or StL has 100% activity at 0 mM taurocholate for the native state at pH 8.0 and 37 ° C. The grey zone indicates the micellar region of TC. The error on the activity determinations is 7–12%.

3.3. Stl shows improved activity compared to TlL in bile salts

Our observations indicate that the main challenge in the use of TlL as a dietary supplement is its poor refolding ability under weakly acidic conditions. Protein engineering may potentially improve stability and activity efficiency of enzymes [39–43]. We therefore selected the variant StL which in other contexts has been shown to be more stable than TlL. StL is a composite mutant based on single-point mutations which individually have led to significant increases in thermal stability and/or enzymatic activity in the presence of anionic surfactants (Novozymes A/S, unpublished). Like TlL, native StL is activated in bile salts over the pH range 4–8 with an optimal value at 0.5–1 mM bile salts (Fig. 1D–F). However, the refolding yields at pH 6 and 4 are clearly much better than for TlL.

To simulate the variation in gastric pH, we investigated the effect of starting pH (range 1.0–5.0) on the recovery of TlL and StL activity and Trp fluorescence at a final pH of 6.0 in 5 mM TC (Fig. 3A and B).

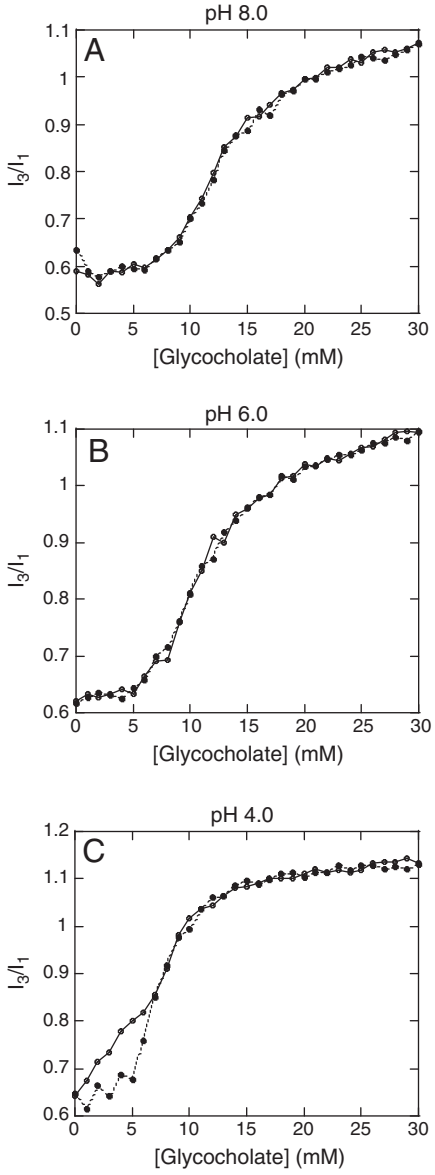

Fig. 2. (A–C) TlL-glycocholate interactions measured with fluorescence at pH 4–8. The pyrene I_3/I_1 ratio reports on the formation of micelles in the absence and presence of 1 μM TlL.

Both activity and TF show that StL completely recovers activity and structure from pH 2.4 onwards (50% recovery around pH 2.0); the corresponding values for TlL are pH 3.0 and pH 2.6, respectively. Unlike TlL, the Trp fluorescence of StL decreases upon transfer to pH 6 from lower pH in the presence of bile salts, but this most likely reflects changes in the Trp environment and its consequent fluorescent properties due to the many positive charges introduced into StL.

3.4. StL shows an improved pH stability profile at low pH compared to TlL

To complement this work, we sought direct evidence that StL can maintain its native structure at lower pH than TlL. This evidence can be obtained by several different techniques. Direct evidence for the shift in native versus denatured populations may be obtained by SDS-PAGE [44,45]. We have already shown that native TlL is stable at pH 8 in 10 mM SDS and migrates differently compared to denatured TlL [29]. Samples incubated at pH 2–8 before running on SDS-PAGE reveal a band shift ≤ pH 2.8 and below, indicating that TlL has lost its native structure at these pH values (Fig. 4A and B). There is almost no native population of TlL below pH 2.4. These results were confirmed by Trp fluorescence and CD spectra which showed denaturation midpoints very similar to those from SDS-PAGE (Fig. 4C and D). StL's fluorescence and CD spectra change in the same way as TlL as they both start to loose structure at low pH, but the transition is shifted down by 0.6–0.8 pH units. TlL starts to unfold at pH 2.8 at 37 °C and is completely denatured below pH 2.4, while StL starts to unfold at pH 2.2 at 37 °C and is completely denatured below pH 1.6.

3.5. Stl is intrinsically more stable than TlL

To ascertain whether StL's superior behaviour in bile salts is strictly related to pH stability or reflects a more general property of the protein, we measured unfolding kinetics of StL in the chemical denaturant guanidinium chloride (GdmCl) at different pH values using Trp fluorescence. Given that GdmCl reacts only weakly and unspecifically with proteins [46], the stability of StL in GdmCl will reflect intrinsic protein properties rather than pH stability. TlL unfolds extremely slowly in GdmCl, making it experimentally impossible to obtain complete equilibration between native and denatured TlL at all GdmCl concentrations and thus ruling out simple equilibrium denaturation experiments [29]. Instead we use unfolding kinetics to compare kinetic stability of TlL and StL. Unfolding time profiles can be fitted to single exponential decays, whose unfolding half lives scale logarithmically with GdmHCl concentration (Fig. 5, summarized in Table 1). Clearly StL is more kinetically stable than TlL in GdmCl at all pH values, with a difference of around 1.5 log units (corresponding to ~2 kcal/mol) between k_{unf} for TlL and StL at all pH values. StL (t_m 80 °C) also has higher thermal stability than TlL (t_m 72 °C) (Fig. S4A and [28]).

3.6. Stl is more kinetically stable in SDS than TlL

TlL does not follow microscopic reversibility at neutral pH values in the presence of surfactant, that is, it does not reach the same structural/functional state from different starting conditions in the experimentally accessible time scale [29]. To test whether this is also the case for StL, we incubated StL at a range of SDS concentrations, starting out from conditions where the protein is initially either in the native state or in the denatured state. We titrated native StL with SDS in 50 mM buffer at different pH values and monitored the reaction using Trp fluorescence. TlL's ability to resist SDS denaturation decreases with decreasing pH [29], and this is also seen for StL. Like TlL at pH 2 and 4, StL's Trp fluorescence decreases with increasing [SDS] without any native baseline region, indicating a low level of resistance against SDS denaturation (data not shown). At pH 6–10 (Fig. 6A, B and C), there is a small but

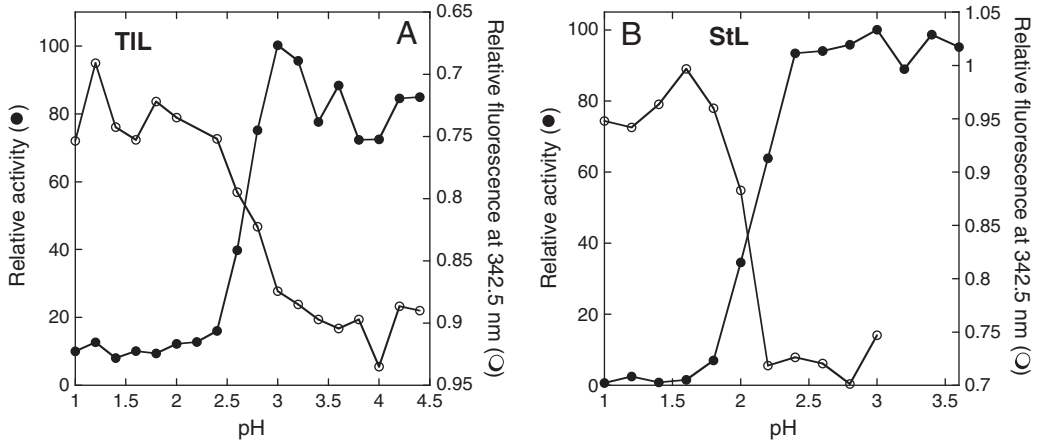

Fig. 3. Starting pH effect on recovery of (A) TlL or (B) StL activity and structure at pH 6 in taurocholate. Proteins were initially incubated in 10 mM buffer pH 1–5 for 1 h, then incubated in 50 mM MES pH 6 and TC for 5 min prior to measuring activity and fluorescence. Trp fluorescence and activity values were normalised to a value of 1.0 and 100% at the highest level, respectively. Individual errors on activity determinations and Trp fluorescence are ~10 and 5%, respectively.

significant increase in Trp fluorescence around 0–2 mM SDS, followed by a plateau and a decline, but the fluorescence of StL decreases much less than that of TlL at pH 8 (Fig. 6B). Far-UV CD spectra at pH 8.0 (Fig. 6D) confirm native-level secondary structure in SDS. Both Trp fluorescence (Fig. 6E) and activity assays (Fig. 6F) reveal that StL's native state is lost significantly more slowly than that of TlL at 25 °C. With half-lives of 202 versus 5 h (cfr. legend to Fig. 6F), the loss of activity by StL occurs ~40 times more slowly than for TlL. Further data supporting the increased stability of StL compared to TlL is provided in the supplementary information, which reveal that TlL has lower

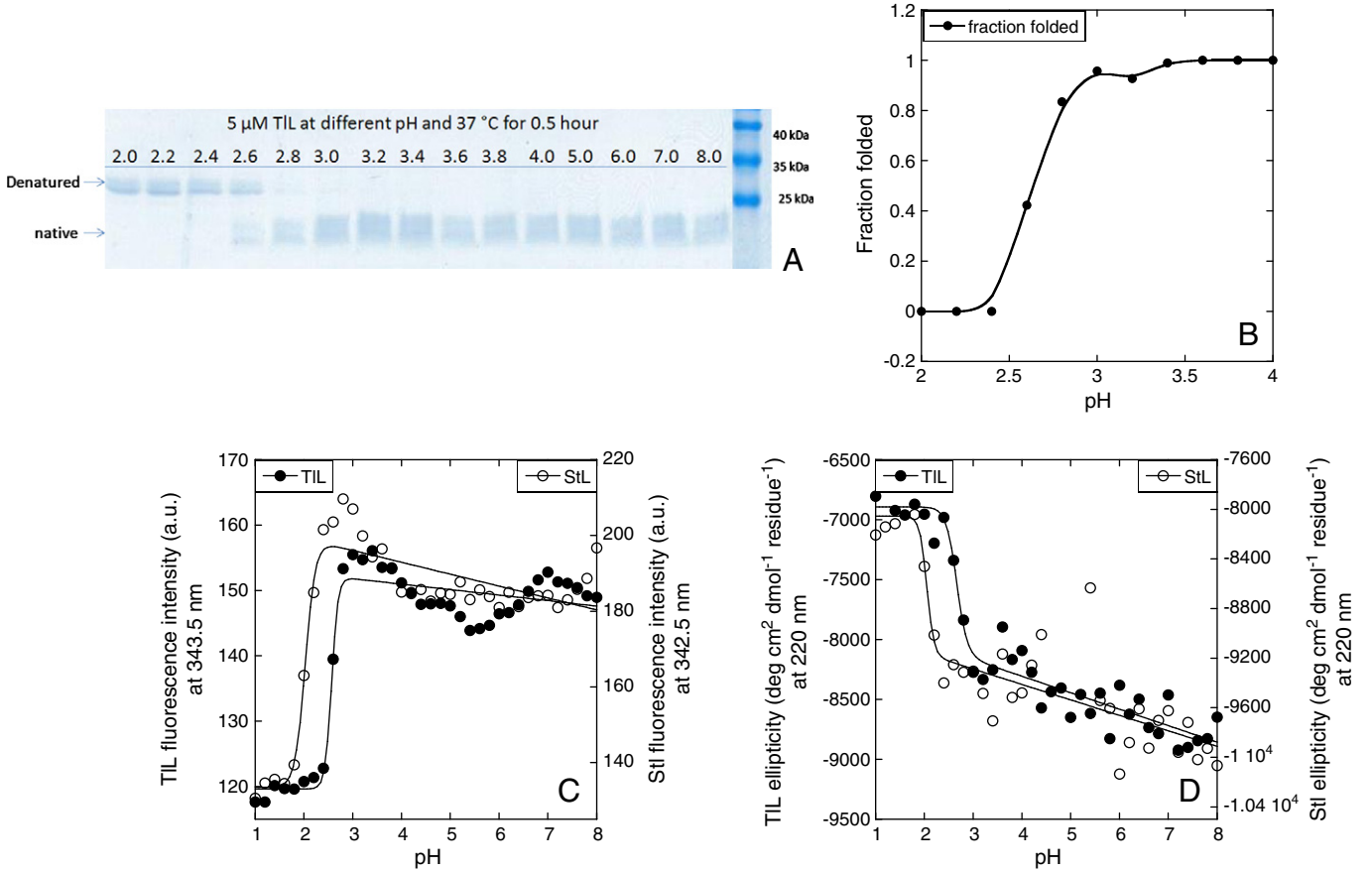

Fig. 4. Assay of pH stability of TlL and StL. (A) SDS-PAGE assay of TlL pH stability. TlL was initially incubated in 10 mM buffer pH 2–8 at 37 °C for 0.5 h, then mixed with loading buffer containing 2% SDS at pH 8 and run without prior boiling. (B) Plot of fractions of native TlL band versus pH based on band intensities from panel A. (C) Fluorescence of TlL and StL at pH 1–8. (D) Ellipticity of TlL and StL at pH 1–8. Data from panels C and D fitted to a sigmoidal function corresponding to a two-state denaturation [62] to guide the eye and estimate the midpoint of the transition.

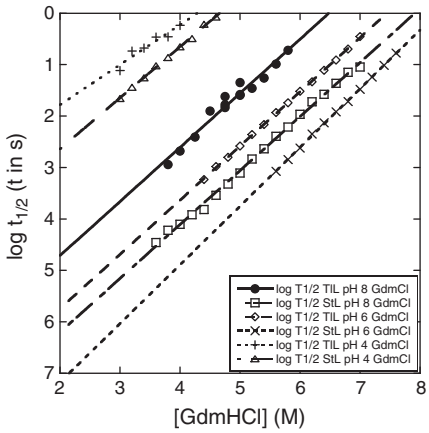

Fig. 5. (A) StL or TlL [29] unfolding half-lives as a function of GdmCl. Linear fits extrapolated to 0 M GdmCl indicate the unfolding half lives in buffer at pH 8, pH 6 and pH 4 (Table 1).

thermal stability than StL in SDS and faster unfolding rates at pH 6 (measured at 25 °C, Fig. S4B) and at pH 8 (measured at 50 °C, Fig. S4C).

TlL's ability to refold from the pH- and urea-denatured state (pH 2.0 and 10 M urea) upon dilution of urea and transfer to pH 8–10 diminishes markedly in the presence of surfactant [29]. This effect is even more pronounced for StL refolding in surfactant. StL and TlL refolding are completely obstructed at concentrations above 3 and 5–6 mM SDS, respectively (Fig. 6B). Thus the hysteresis in fluorescence intensity between native and refolded StL is even more pronounced than that of TlL (Fig. 6B).

4. Discussion

4.1. Weak interactions between TlL and bile salts compared to interactions between TlL and SDS

In the present study, we found that bile salts can trap the low-pH-treated TlL in a denatured state at pH 4 and pH 6 and even promote precipitation of native TlL at pH 4. Furthermore, both bile salts activate native TlL significantly at low concentrations and inhibit it at higher concentration, consistent with a double-binding activation–inhibition scheme previously discussed for TlL in the context of simpler surfactants, involving a combination of general and structurally specific interactions [28]. However, bile salts do not destabilise native TlL as they do not affect its conformation, heat stability or resistance to proteolysis. Furthermore, no interactions between TlL and bile salts or between substrate and bile salt were detected by ITC, indicating that these interactions are rather weak. When TlL is incubated at low pH, it loses its native structure [29]. This presumably exposes hydrophobic regions which may bind to bile salts, trapping it in a denatured state in a pH-dependent manner, since the level of activation increases with the refolding pH.

Table 1
Half-lives of unfolding of TlL and StL in buffer at pH 4–8.

Variant	$t_{1/2}^{0M\ GdmCl}$ (days) [a]		
	pH 8.0	pH 6.0	pH 4.0
TlL[b]	76.35	820	0.025
StL	2339	33,426	0.487

[a] Extrapolated from unfolding kinetics in high concentrations of GdmCl. Based on Trp fluorescence monitored over time.
[b] From [29].

SDS has a much stronger interaction with TlL than bile salts, according to spectroscopy as well as isothermal titration calorimetry [29]. The difference between SDS and bile salts is that SDS has a more highly charged acidic head group and a flexibly alkyl tail. This facilitates the binding to positively charged side chain on the protein surface and hydrophobic core of protein. With most proteins this results in denaturation at low SDS concentrations [47], except for kinetically stable proteins [44,45]. In contrast, the bile salts can emulsify fat and activate pancreatic lipases without denaturing or inhibiting the enzyme, though other lipases such as that from *R. arrhizus* are inhibited [19]. This effect may be related to its unusual molecular structure, namely a rather weak acidic head group and two or three hydroxyl groups attached to a rigid hydrophobic face [48]. The large and rigid hydrophobic steroid moiety of bile salts most likely binds to hydrophobic parts of proteins, while the hydrophilic groups presumably contact hydrophilic parts of protein or remain water-exposed. Hydrophobic interactions with TlL could involve the region around the substrate-binding site of native TlL or contiguous regions found in TlL's denatured state, trapping the protein in a non-native state.

Many different types of protein–bile interactions are possible, and this leads to many different effects, ranging from dissociation of insulin oligomers without denaturation [49] to a dramatic enhancement of the proteolytic susceptibility of β-lactoglobulin, bovine serum albumin and myoglobin [50]. There may also be indirect effects. Low concentrations of bile salts prevent the interfacial denaturation of lipases from *Y. lipolytica* (YLLIP2) as surfactant monomers lower the interfacial tension [19,22]. Further progress in our understanding of these interactions will benefit from structural information on protein:bile complexes provided by *e.g.* Small Angle X-ray Scattering (SAXS). This technique has already demonstrated how proteins can stabilise micelles well below the cmc [51,52]. Given the rigid structures of bile salts and the low number of bile molecules required to form micelles, we may expect coming structures to reveal a number of idiosyncratic features which reflect specific binding features.

4.2. The stability of TlL can be significantly increased by genetic engineering, leading to improved activity in bile salts

In an application-relevant context, enhanced activity in bile salts over a wide pH range is likely beneficial as it implies that TlL will display significantly increased activity and stability at intestinal pH (typically 4.0–7.5 [12,13]). The activation of both native and low-pH-treated TlL at low bile concentrations also favour the application, as pancreatic insufficiency usually leads to less than 1 mM bile salt [8]. Clearly TlL and StL are not affected by TC as long as they are native when exposed to TC. Only if the protein is denatured and then refolded in the presence of TC is it prevented from gaining activity. StL can tolerate pH down to 1.8 while TlL can only tolerate pH 2.6, suggesting Stl could overcome the low pH at the stomach-intestine interface (duodenum). The unfolding rates of StL in buffer, extrapolated from high concentrations of GdmCl, are consistently log 1.5–32 times longer than wild type TlL at pH 4–8. Kinetic stability in surfactant is also greatly increased, with an activity half-life of 9 days in SDS for StL at neutral pH 8 while it is 2 h for TlL. The long half-lives are comparable to those obtained for other conserved digestive enzymes [44]. Thus StL behaves as a better candidate than TlL with regards to enzyme replacement therapy in connection with a harsh digestive environment.

What could be the reason for StL's enhanced stability? Compared to TlL, StL has 4 more positively charged residues and one less negatively charged (A.S. and K.B., data not shown). This increases the pI from TlL's 5.0 to an estimated 6.1 (theoretical calculations of the overall charge shown in Fig. 7A). We have modeled the StL structure based on the known TlL X-ray structure. Overall the mutations make the electrostatic potential more positive over the entire pH range (Fig. 7B). Molecular dynamics simulations show considerably reduced mobility

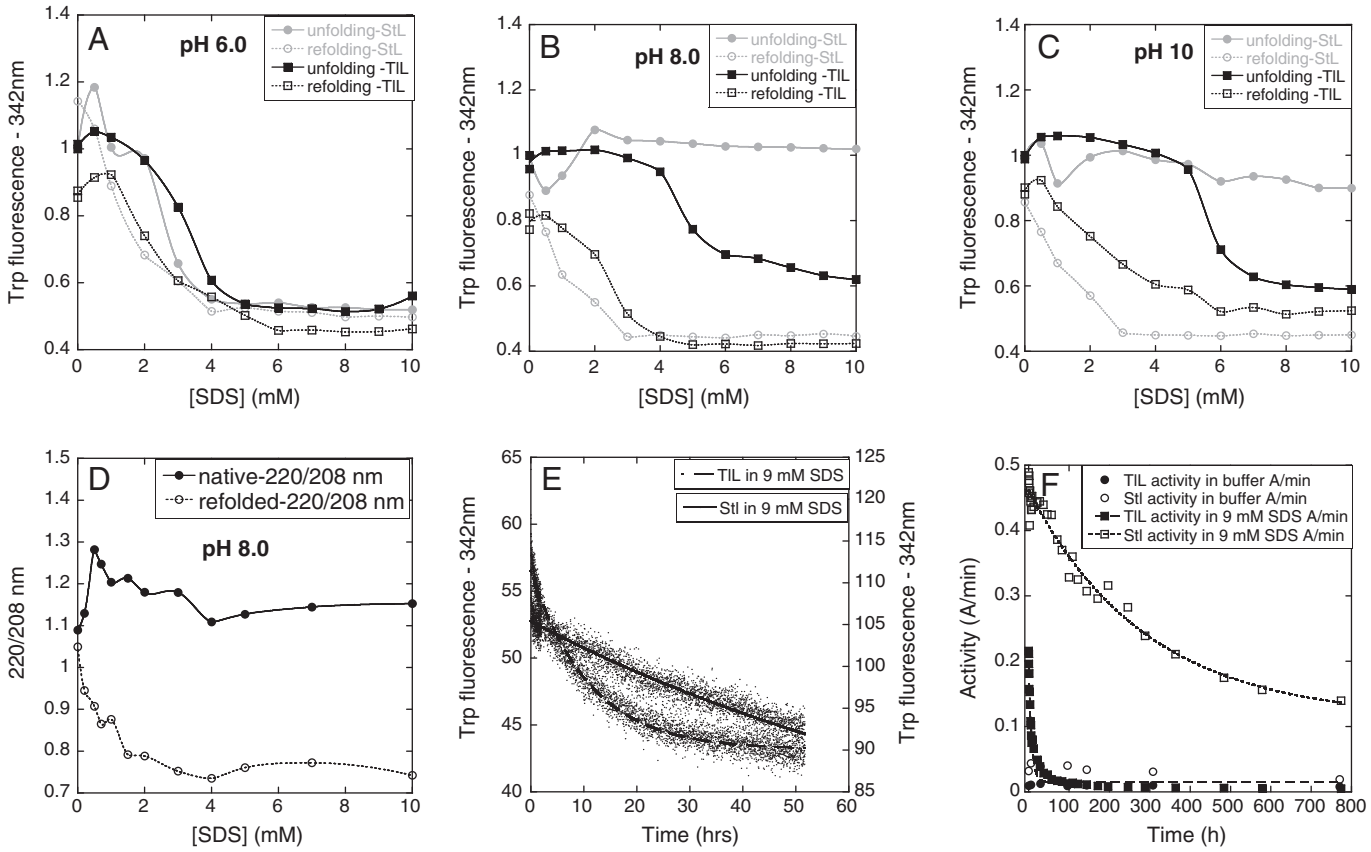

Fig. 6. (A–D) StL-SDS interactions measured with Trp fluorescence and ellipticity (220 nm/208 nm ratio) as a function of SDS concentration. B also includes TIL-SDS interaction measured with fluorescence at pH 8 from [29] for comparison. Solutions were incubated for 24 h prior to measurement. Trp fluorescence units were normalised to a value of 1 at 0 mM SDS for the native state at each pH. (E and F) Kinetics of change in Trp fluorescence and activity for TIL or StL at 0 and 9 mM SDS at pH 8.0. Data are fitted to single exponential decays. In E, the half lives of the exponential decay is 7.6 ± 0.1 h for TIL but was too slow (well above 50 h) to be determined accurately for StL. In F, the half-lives are 5.0 ± 0.4 and 202 ± 26 h for TIL and StL, respectively.

for StL compared to Wt, which is a good indication for higher stability (A.S, data not shown). This can be explained by the fact that removal of a negative charge (and thus overall increasing the positive charge) optimizes a local hydrogen-bonding network and reduces mobility. In addition to this, the overall introduction of positive charge may have a beneficial effect. Increased positive charge has led to increased stabilization of firefly luciferase [53], streptococcal protein G [54] and *A. acidocaldarius* maltose-maltodextrin-binding protein [55]. Clusters of positively charged residues together with aromatic residues can also form strong hubs which help bring together different secondary structural elements in the tertiary structures of the proteins and thus contribute to enhanced stability [56].

SDS denatures TIL by nucleating via a critical number of bound SDS molecules on the surface of the native protein to form clusters [29]. *A priori*, StL would be expected to be less stable in SDS since Stl has four more positive charges and one less negative charge. However, this potential effect is overruled by the increased intrinsic/kinetic stability, which leads to ~32-fold lower rates of unfolding of StL compared to TIL in GdmCl. In fact, there is also a factor ~40 difference between the two proteins' unfolding rates in SDS. Clearly StL has a significantly higher barrier to unfolding than TIL. This will also lead to a greater activation barrier to refolding (provided the stability of the denatured state is not affected), and agrees well with the greater level of hysteresis exhibited by StL in SDS.

TIL and StL are quite acid- and protease resistant and are only digested by pepsin if the protein is denatured at a pH sufficiently low to denature the protein, *i.e.* pH 2.6 for TIL and pH 1.8 for StL (data not shown). These pH values are found under some conditions in the stomach [7,13]. Further improvements in acid stability may be achieved through additional electrostatic mutations. Alternative solutions include coating with acid-resistant polymers, leading to slow release at a safe pH value. This approach has been used for porcine pancreatic enzymes [57,58]. However, human gastric lipase, which had been assumed to be the most acid stable enzyme among the pre-duodenal lipases, is also irreversibly inactivated in gastric juice at pH values below pH. In the absence of gastric juice, purified human gastric lipase is inactivated at pH 3.0 with a half-life of 25 min, suggesting that human gastric lipase is probably stabilised by other components of the gastric juice, such as the mucus, under physiological conditions [59]. Moreover, proteolytic degradation of human gastric lipase by pepsin also occurs at pH 1.0, probably because the acid-denatured human gastric lipase is unfolded and exposes proteolytic cleavage sites to pepsin [59]. Assuming that human gastric lipase is also be hydrolysed by human pepsin at pH 2.0 (cfr. its short half-life at pH 3.0), we conclude that StL is even more stable than the purified human gastric lipase, since we have shown that StL is able to survive at pH 2.0 in the presence of pepsin. StL's improved acid tolerance will clearly benefit patients with exocrine pancreatic insufficiency, whose duodenal pH is ≤ pH 4, leading to inactivation of porcine lipase [60,61].

Acknowledgements

H.W. was funded by the Danish Research Training Council, Abbott and Novozymes A/S. D.E.O. is supported by the Danish Research Foundation (inSPIN) and the Lundbeck foundation (BioNET 2).

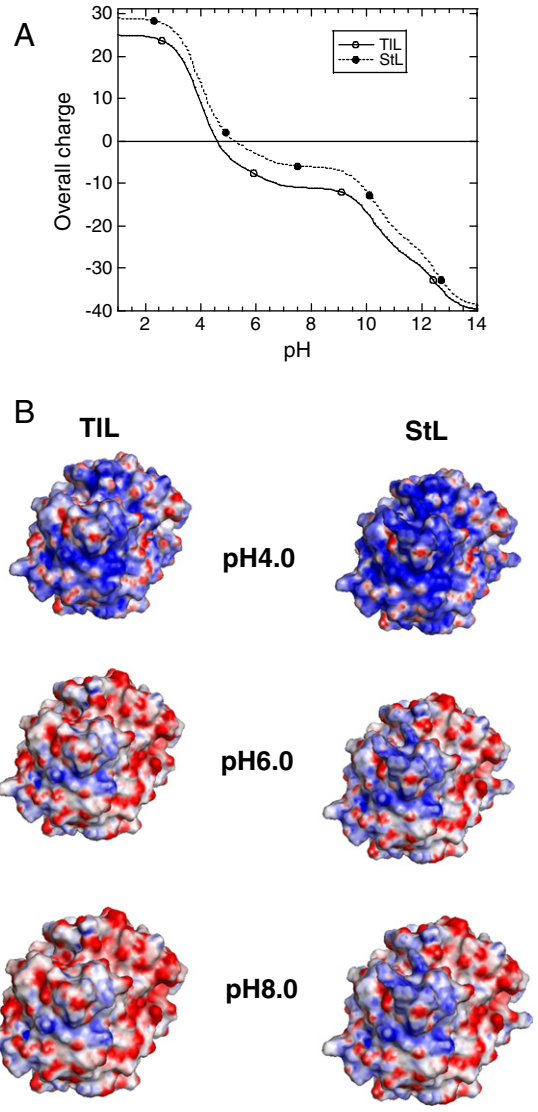

Fig. 7. (A) Theoretical pH-titration of TlL and StL show an increase in overall positive charge of StL. (B) Electrostatic potential of TlL and StL at pH 4, 6 and 8.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bpc.2012.12.003.

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