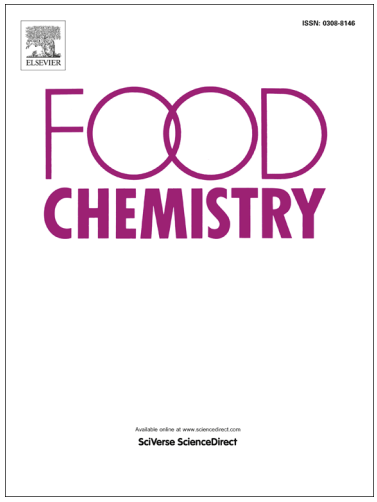

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Effects of temperature, pH and sugar binding on the structures of lectins Ebulin f and SELfd

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ABSTRACT

Ebulin f and SELfd are two lectins of Sambucus ebulus L. that show different stability and digestibility properties in gastric fluid due to their structural differences which may explain their different toxicological profiles.

The main aim was to determine the effects of pH, temperature and sugar binding on the intrinsic structures of both proteins by fluorescence analyses.

Quenching experiments were conducted, under different pH and temperature conditions, with acrylamide (uncharged) and iodide (charged), to study the possible changes of their intrinsic fluorescence.

Results revealed that the native structure of SELfd is more folded than that of ebulin f. At pH 2.0, ebulin f displayed a more open structure than at neutral pH.

It can be concluded that this is the main reason why ebulin f is accessible to pepsin action and more sensitive to degradation, in contrast to SELfd as we reported previously.

KEY WORDS

Ebulin f; SELfd; pepsin, lactose; D-galactose; lectin; *Sambucus ebulus*; dwarf elder.

Chemical compounds studied in this article

Sepharose (PubChem CID: 11966311); hydrochloric acid (PubChem CID: 313); acrylamide (PubChem CID: 6579); guanidinium chloride (PubChem CID: 5742); lactose (PubChem CID: 84571); D-galactose (PubChem CID: 6036); 8-anilino-1-naphthalenesulfonic acid (PubChem CID: 24890494).

1. Introduction

Lectins are proteins with sugar-binding ability found in all kinds of organisms, some being cell-agglutinating proteins (Sharon, 2008). Special types of lectins are those which contain an enzymatic activity that catalytically and irreversibly inhibits protein synthesis. They are thus known as ribosome-inactivating proteins (RIPs). Lectins of this type can interact with the polysaccharides present in cell epithelial receptors and induce toxicological effects (Schrot, Weng & Melzig, 2015).

The carbohydrate binding property of lectins accounts for their use as a biomarker in cancer research (Dai, Zhou, Qiu, Liu & Fan, 2009). RIPs of certain species of plants and bacteria are good candidates as toxic moieties in the construction of immunotoxins and conjugates directed against specific targets (Stirpe, 2013; Gilabert-Oriol, Weng, Mallinckrodt, Melzig, Fuchs & Thakur, 2014). Lectins have also been described as allergens in food (Wickham, Faulks & Mills, 2009). Protein allergenicity seems to be related to its integrity, which is affected by conditions, such as temperature, pH and protein structure (Astwood, Leach & Fuchs, 1996; Dimitrijevic, Jadranin, Burazer, Ostojic & Gavrovic-Jankulovic, 2010; Jimenez, Cabrero, Tejero, Basterrechea, Cordoba-Diaz & Girbés, 2013a). Since there is no general rule on the issue, a detailed characterization of the protein structure for each individual case is recommended to establish a protein's allergenicity.

Dwarf elder (Sambucus ebulus L.) is a medicinal plant, used since ancient times for a number of disorders. Among them are wound healing, anti-dermatitis (Ebrahimzadeh, Rafati, Damchi, Golpur & Fathiazad, 2014), antidepressant (Mahmoudi, Ebrahimzadeh, Dooshan, Arimi, Ghasemi & Fathiazad, 2014), antiemetic (Fathi, Ebrahimzadeh, Ziar & Mohammadui, 2015), anti-giardial (Rahimi-Esboei, Ebrahimzadeh, Gholami & Falah-Omrani, 2013), anti-inflammatory (Jabary, Hashempur, Razavi, Shahraki, Mamalinejad & Emtiazy (2016).

Specific effects of fruit infusion on lipid metabolism by improving lipid profile and increasing the serum antioxidant capacity in healthy volunteers (Ivanova, Tasinov, Kiselova-Kaneva, 2014), have been described. The powerful antioxidant properties have been reported (Kiselova, Ivanova, Chervenkov, Gerova, Galunska & Yankova, 2006), and its usefulness as a food and medicinal supplement has been suggested (Jimenez et al., 2013a). These above described properties make dwarf elder a candidate for developing standardized preparations for the pharmaceutical industry.

Unripe *Sambucus* fruits, particularly those from dwarf elder, show an, as yet, clearly undefined toxicity. They contain a fruit form of ebulin (ebulin f) and a related lectin (SELfd). Ebulins have two chains: the A chain, with N-glycosidase activity on 28S rRNA, responsible for the irreversible inactivation of ribosomes, and a B chain, with D-galactose-specific binding activity. They are therefore known as type 2 (two chains) RIPs (Ng, Wong & Wang, 2010; Schrot, et al., 2015). Together with type 2 RIPs, fruits from *Sambucus ebulus* L. contain an ebulin-related lectin of the B-B type (SELfd) which is devoid of translational inhibitory activity and only exhibits D-galactose-specific binding (Schrot, et al., 2015). In a previous report, both ebulin f and SELfd were subjected to analysis by MALDI-TOF mass spectrometry, tryptic peptide fingerprinting, molecular characterization and sensitivity to simulated gastric fluid (Jimenez, Tejero, Cabrero, Cordoba-Diaz & Girbés, 2013b). Ebulin f was more sensitive to hydrolysis than was SELfd. We recently found that ebulin-blo, an isoform of ebulin isolated from blossoms, is toxic upon oral ingestion and attributed this to poor digestion which might allow the toxin to enter the small intestine and to cross the intestinal barrier (Jimenez, et al., 2013a). The differential sensitivity of the two proteins to simulated gastric fluid was thought to be related to their tertiary structure. It has been found that pepsin sensitivity of lectins suffers conformational alterations promoted by preheating,

which significantly increases its sensitivity to pepsin (He, Simpson, Ngdi & Ma, 2015; Jimenez, et al., 2014).

The fluorescence properties of proteins have been used extensively for such a purpose. The intrinsic fluorescence of a protein is conferred by aromatic residues such as tryptophan (Trp), tyrosine and phenylalanine (Lakowicz, 2006). Fluorescence of Trp is influenced by its microenvironment. Thus, changes affecting the tryptophan environment alter the fluorescence properties of proteins. These changes in the intrinsic fluorescence have been used to gain an understanding of the proteins' structure, specificity and conformation (Khan, Ahmad & Khan, 2007). The aim of the present work was to gain insights into the conformational stabilities of ebulin f and SELfd, and to explore the effects of pH, sugar binding and temperature on native structure, by fluorescence analysis, in order to explain their differential pepsin sensitivities seen in a previous report from our laboratory (Jimenez et al., 2013b).

2. Materials and methods

2.1. Materials

Chromatographic supports were purchased from Pharmacia Ibérica (Madrid, Spain). Acid-treated Sepharose 6B was prepared by treatment of Sepharose 6B beads with 0.1 N HCl at 50ºC for 3 h and extensive washing with Helix Millipore water (Jimenez et al., 2013b). Green fruits of dwarf elder (*Sambucus ebulus* L.), harvested in Barruelo del Valle (Valladolid, Spain) in early July, were stored frozen at -20ºC. The average weight of the fruits was approximately 6.5 mg/fruit from the same bush. Acrylamide, guanidinium chloride (Gdn.HCl), lactose and D-galactose, were purchased from Sigma-Aldrich Quimica SL (Madrid, Spain). All other common chemicals and bio-chemicals were of the highest purity available.

2.2. Isolation of D-galactose-binding proteins from the green fruits of dwarf elder

200 g of wet green dwarf elder were ground in a blender for 2 min to obtain a finely cut material. The minced material was extracted overnight with 800 ml of extraction buffer (280 mM NaCl containing 5 mM sodium phosphate pH 7.5). The extract was strained through two layers of cheesecloth and the fluid was then centrifuged at 7,500 *g* for 30 min at 4ºC, after which the supernatant was centrifuged again at the same speed for 30 min, removed, filtered through two layers of filter paper and stored at 4ºC for a short time until used; 650 ml of extract were applied to a XK50 (5 x 15 cm) column (GE-Pharmacia) containing 200 ml of acid-treated Sepharose 6B (AT-Sepharose 6B) equilibrated with 0.28 M NaCl and 5 mM Na-phosphate (pH 7.5) buffer. The column was then washed with the same buffer until the absorbance values at 280 nm reached values lower than 0.1, and the protein fraction not retained by the AT-Sepharose 6B column was discarded. The retained protein fraction was further eluted with the same buffer, now containing 0.2 M lactose. Fractions of 10 ml were collected and those containing proteins were pooled and concentrated with an Amicon system, using a Y10 membrane to 5 ml. This protein solution was applied to a Superdex 75 (26/60; GE) column equilibrated with 400 mM NaCl and 5 mM Na-phosphate (pH 7.5) buffer. The column was then eluted with the same buffer at 2.5 ml/min. Fractions of 2.5 ml were taken and their A_{280} was measured, and those containing proteins were pooled. Finally, all fractions containing the separated lectins were dialyzed against water and finally concentrated with an Amicon system (Y10 membrane) at 2.5-4.0 mg/ml, divided into aliquots of 0.1 ml, and stored frozen at -20ºC. The purity of both proteins was assessed by SDS-PAGE.

2.3. Quenching fluorescence measurements

Fluorescence measurements were carried out using a Cary Eclypse spectrofluorometer (Agilent Technologies S.L., Las Rozas, Spain), with a slit width of 5 nm for both monochromators and a scan speed of 100 nm/min. Titration of the proteins (1 μM) with acrylamide and KI was performed in the absence and presence of Gdn.HCl (6 M) (conditions A and B). The iodide solution contained sodium thiosulfate (200 μM) to suppress tri-iodate formation. Fluorescence emission spectra were recorded after 3 min and each spectrum was an average of three accumulations. The excitation wavelength was 295 nm. All measurements were performed at 25ºC. For sugar-binding studies, proteins were prepared under saturating concentrations of lactose and D-galactose (0.1 M) (conditions C and D).

Quenching data for all the quenchers used in this study were analyzed by the Stern-Volmer Eq. 1, as well as by the modified Stern-Volmer Eq. 2 (Lehrer, 1971)

$$F_o/F_c = 1 + K_{SV}[Q] \quad (1)$$

$$F_o/\Delta F = f_a^{-1} + 1/(K_a \cdot f_a[Q]) \quad (2)$$

where F_o and F_c are the respective fluorescence intensities, corrected for dilution, in the absence and in the presence of a quencher, [Q] is the resultant quencher concentration, K_{SV} is the Stern-Volmer quenching constant, ΔF (F_o-F_c) is the change in fluorescence intensity at any point in the quenching titration, f_a is the fraction of all the fluorophores accessible to the quencher and K_a is the corresponding Stern-Volmer association constant for the fraction of the fluorophores.

2.4. Temperature and pH-dependence studies

In thermal treatment experiments, the spectra were recorded for 10 minutes after the desired temperature (4, 10, 15, 20, 25, 30, 40, 50, 60, 80, 90 ºC) was attained and each spectrum was

an average of three determinations. For refolding studies, the protein was heated to the desired temperature and cooled to 25ºC to record the emission spectra.

For pH-dependence assays, the protein was prepared in different phosphate buffers: pH 2.0, pH 7.5, pH 10.5 and pH 13.0, and fluorescence emission spectra were recorded at 25°C. When the joint pH-temperature effect was studied, each sample was heated at a range of 0-90ºC in the different buffers, as described for temperature measurements.

3. Results and discussion

3.1. Fluorescence quenching studies

Lectins from *Sambucus* species have been purified from several species and some have been characterized (Tejero, et al., 2015; Jiménez, et al., 2015). In the present study, we investigate tryptophan exposure and microenvironment of two lectins isolated from dwarf elder fruits (*Sambucus ebulus* L.) by quenching the protein intrinsic fluorescence. Acrylamide (neutral molecule) and iodide (anion) were used as quenchers. It is well known that acrylamide can partially penetrate into the folded protein matrix and therefore quench the fluorescence of even partially buried Trp residues. By contrast, iodide can only quench surface-exposed Trp residues (Eftink & Ghiron, 1976). Due to the relevance of the results, we only show graphic data on acrylamide quenching.

Fluorescence spectra of ebulin f and SELfd in the native state and upon denaturation with 6 M Gdn.HCl, recorded in the absence and presence of different concentrations of acrylamide, are shown in Fig. 1. Spectrum 1 corresponds to the lectin alone, while the spectra numbered 2-20 correspond to those recorded in the presence of increasing concentrations of the quencher. Spectra in Fig. 1 also show that SELfd (homodimeric lectin of 614 amino acids with 7

tryptophan residues per monomer; Jimenez, et al., 2013b) has a maximum of around 334 nm while native ebulin f (heterodimeric lectin of 564 amino acids and 10 tryptophan residues) exhibits an emission maximum at 341 nm. Both proteins showed a red-shift to 356 nm upon denaturing conditions. Quenching by acrylamide is higher with the denatured lectin than with the native lectin, and similar results were observed for iodide (spectra not shown). Our data suggest that SELfd has its Trp in a more hydrophobic environment than has ebulin f. Upon denaturation with 6 M Gdn.HCl, a large red shift in the emission maximum of the protein was observed, indicating a great exposure of the tryptophan residues to the aqueous environment and suggesting that the protein is unfolded. Similar results have been obtained with several seed lectins from *Cucurbitaceae* species (Sultan, Kavitha & Swamy, 2009). Upon denaturation, all Trp residues are exposed to the aqueous media and therefore the emission λ_{max} of both lectins increases and they are centred between 350 and 360 nm according to previous descriptions (Lakowicz, 2006).

The degree of quenching achieved at a resulting concentration of 0.5 M of acrylamide, under different conditions, is given in Table 1. Binding of lactose and D-galactose to ebulin f results in a blue-shift of the λ_{max} emissions to 336 and 338 nm, respectively. However, λ_{max} remains the same for SELfd after sugar-binding (data not shown). Results obtained for iodide are included in Table I as supplementary material. Sugar binding to the protein might be expected to shield those Trp residues involved in the sugar-binding site, thus reducing their accessibility and leading to a subsequent decrease in the extent of quenching. SELfd fluorescence quenching by acrylamide and I^- did not significantly change in the presence of either lactose or D-galactose. In addition, neither of the sugars changed the emission λ_{max} of the lectin. Accordingly, our results suggest that Trp residues do not seem to be directly involved in carbohydrate binding by SELfd. By contrast, binding of lactose and D-galactose to ebulin f triggers a blue-shift in the emission λ_{max} resulting from the binding of lactose and

galactose. This suggests that sugar binding leads to a tightening of ebulin f structure that makes the Trp residue environment less accessible to the aqueous medium. This is also consistent with the decrease in quenching by acrylamide and iodide observed in ebulin f. Differing results may be found in the literature. A blue-shift from 338 to 335 nm has been observed for a chitooligosaccharide-specific pumpkin (*Cucurbita maxima*) phloem exudate lectin (PPL) in the presence of saturating concentrations of chitotriose, suggesting that ligand-binding leads to a decrease in the solvent exposure of the tryptophan residues (Narahari & Swamy, 2009). By contrast, fluorescence studies of *E. speciosa* seed lectin (EspecL) indicated that tryptophan residues were present in a highly hydrophobic environment, and that binding of lactose to EspecL neither quenched tryptophan fluorescence nor altered λ_{max} position (Konozy, Bernardes, Rosa, Faca, Greene & Ward 2003).

Stern-Volmer plots obtained with both acrylamide (Fig. 2A) and iodide (data not shown) were biphasic for ebulin f, and monophasic for SELfd. The presence of saturating concentrations of lactose or D-galactose also yielded biphasic Stern-Volmer plots with acrylamide in ebulin f. By contrast, no significant change in Stern-Volmer plots observed for SELfd was seen in the presence of either sugar. Interestingly, under denaturing conditions, the Stern-Volmer plot for acrylamide was linear at low quencher concentrations, but showed upward curvature at higher concentrations of the quencher (Fig. 2A). Further, the Stern-Volmer plot for iodide ion quenching becomes linear, indicating that all the tryptophan residues quenched have comparable accessibilities (data not shown). From the slopes of these plots, the corresponding Stern-Volmer constants (K_{SV1} and K_{SV2}) were obtained and are listed in Table 1. The K_{SV} values obtained indicate that acrylamide is significantly more efficient than iodide ion in quenching the intrinsic fluorescence of ebulin f and SELfd. Binding of lactose and D-galactose increases the K_{SV1} value for acrylamide quenching but decreases $K_{SV2.}$ In contrast, quenching by the iodide ion decreases both constants (data not shown).

These observations are consistent with the results expected from dynamic fluorescence quenching of proteins with two or more Trp type residues in different microenvironments with different accessibilities to the quenchers (Sultan, et al., 2009). Since ebulin f is an A-B type lectin, it could be hypothesized that the accessibilities of Trp residues of the A and B chains might differ. According to our results (Ksv1), Trp residues located in the A-chain of ebulin f are more accessible whereas, by contrast, Trp residues located in the B-chain would be less accessible (Ksv2). This is supported by the data obtained for SELfd, indicating a lower accessibility of the Trp residues, more or less coinciding with the Ksv2. It must be remembered that the ebulin b B-chain and SELfd B-chains display a large degree of amino acid identity. Under denatured conditions Stern-Volmer plots show upward curvature for both proteins in the case of acrylamide. Such upward curving profiles have been reported for other proteins (Sultan, et al., 2009; Narahari & Swamy, 2009; Katre, Suresh, Khan & Gaikwad, 2008).

Modified Stern-Volmer plots for ebulin f and SELfd with acrylamide (Fig. 2B) and iodide (data not shown), under different conditions, gave a linear response. The fraction of the total fluorescence accessible to the quencher (f_a) and the corresponding Stern-Volmer quenching constants (K_a) were estimated from the Y-intercepts of the plots. The values obtained are listed in Table 1. Denaturation with 6 M Gdn.HCl led to an increase in accessibility close to 95% for acrylamide and the ionic quencher (data not shown), reflecting a large increase in the solvent accessibility of tryptophan residues in the unfolded protein. In the presence of lactose or D-galactose, Stern-Volmer plots for SELfd show marginal changes (within 10% of the accessibility without sugar), in contrast to ebulin f that presents higher Ksv1 values. This indicates that sugar binding shields the accessible Trp in ebulin f. Our findings are consistent with the results obtained for other reported lectins, such as TCA-I isolated from seeds of *T. cordata* (Sultan, et al., 2009).

Overall, our results indicate that there are clear differences between the native structures of SELfd and ebulin f. Such differences are also supported by the differential surface hydrophobicity between proteins, as elucidated by ANS binding assays (preliminary experiments; data not shown). Our findings are consistent with the idea that there are more exposed Trp residues in ebulin f than in SELfd.

3.2. Effect of temperature on native structure

Protein structure is greatly influenced by environmental factors, such as pH and temperature (Lakowicz, 2006). We investigated the effect of different temperatures on the native structure of both lectins. The corresponding fluorescence spectra are shown in Fig. 3A and 3B. The emission intensity (of both proteins) increases with increase in temperature. As indicated above, the maximum emission intensity of SELfd at 25ºC is centred at 334 nm, whilst that of ebulin f appears around 341 nm. Changes in emission maximum of ebulin f and SELfd, as a result of thermal treatment, are shown in Figures 3C and 3D, respectively. The emission maximum of SELfd does not change in the temperature range between 4 and 70ºC (spectra 1-9). At 70ºC (spectrum 10), the emission maximum shifts to 339 nm, indicating minor alterations of the protein structure. However, the emission maximum at 80ºC shifts to 350 nm (spectrum 11), with a significant decrease in emission intensity. This trend continues at 90ºC (spectrum 12). Results observed with ebulin f differ from those of SELfd in that this protein progressively red-shifts the emission maximum from 350 nm when heated to 90ºC. Possibly temperatures higher than 90ºC led to protein denaturation. Changes in the fluorescence properties of ebulin f incubated at different temperatures indicate changes in the protein structure above 40-50ºC with a progressive increase in emission λ_{max}. In contrast, structural changes observed in SELfd below 70ºC are negligible, although a significant structural change takes place at 80ºC, concomitant with a considerable red-shift in the emission λ_{max} together with a significant decrease in emission intensity.

Furthermore, the results obtained with the D-galactose-specific type 2 ribosome-inactivating protein from seeds of *Momordica charantia* lectin (MCL) are consistent with those of ebulin f (Ng, Wong, Li & Yeung 1986). MCL activity is mostly unaffected in the temperature range 4-50ºC, although a sharp decrease is seen between 50 and 60ºC, which might be correlated with changes in the protein structure, as seen by circular dichroism (CD) and fluorescence spectroscopy (Sultan & Swamy, 2005). Previous reports, focussing on the study of the thermal unfolding of legume lectins by monitoring CD signals, have shown that most of the lectins investigated were generally stable to thermal treatment with the midpoint of transition to denaturation between 56 and 92°C (Sultan, Rao, Nadimpalli & Swamy, 2006). Some of these lectins undergo a two-state unfolding transition with the midpoint at 87-92°C, whereas others exhibit a more complex unfolding process, with two separate transitions with midpoints at 56-61°C and 63°C (Reddy, Bharadwaj & Surolia, 1999; Schwarz, Puri, Bhat & Surolia, 1993). These differences seem to exist between ebulin f and SELfd. Our results support the idea that thermal unfolding of ebulin f involves a more complex mechanism than that experienced by SELfd, most probably due to the different structures of the ebulin f A and B chains. Further experiments are required to clarify this aspect.

Refolding of thermally denatured protein was studied by cooling heated samples to 25°C and then monitoring the fluorescence spectra. (Fig. I, supplementary material) shows the emission λ_{max} at 25ºC recorded after each heating treatment. The emission maximum of SELfd at 25ºC remains unchanged after heating to 70ºC. However, ebulin f is not able to return to the emission native maximum once heated to 50ºC, indicating the irreversible nature of the process. Refolding assays also confirm that SELfd is thermally more stable than is ebulin f, being able to return to its native state even after heating to 70ºC. Similar results were reported for a heterodimer lectin TCA-I (Sultan, et al., 2009).

3.3. Effect of pH on native structure

Fluorescence emission spectra of ebulin f and SELfd at different pH are shown in Fig. 4. SELfd fluorescence is not significantly affected by extreme pH, and there is no significant shift in the λ_{max} at either pH 2.0 or 10.5. By contrast, a red-shift from 334 to 339 nm was observed when the protein was incubated at pH 13.0. Different results were observed for ebulin f, which shows a blue-shift at pH 2.0 from 341 to 337 and a concomitant red-shift at pH 13.0 from 334 to 339. The acidic environment alters the native structure of ebulin f with a blue-shift in the emission λ_{max} suggesting an opposite effect in the conformation of the protein when incubated at pH 13. The changes observed for SELfd under the different pH conditions were minor compared to those observed under extreme alkaline conditions. As observed in the thermal treatments, SELfd is also more stable under pH changes. This is consistent with the denaturation studies of a dimeric lectin from the endophytic fungus, *Fusarium solani*, which shows flexibility in tertiary structure with retention of hemaglutinating activity at acidic pH (Khan, et al., 2007).

In contrast to ebulin f, SELfd is highly glycosylated, opening up the possibility that the glycan chains linked to the polypeptide chains might be involved in SELfd's greater stability to pH and temperature than ebulin f. In fact, previous research has shown the profound effect of glycosylation on the conformational stabilization and oligomerization of proteins (Sinha & Surolia, 2007; Mitra, Sharon & Surolia, 2003; Severino, et al., 2010). Furthermore, the non-glycosylated mutant of cathepsin E showed lower pH stabilities than the glycosylated native enzyme (Yasuda, et al. 1999). The findings reported here lead us to suggest that the homodimeric B-B lectin SELfd presents a more folded structure than does the heterodimeric A-B lectin ebulin f. This could be due, in part, to the glycan chain content of this lectin. In this context, it has been reported that glycosylation plays a primary role in facilitating refolding of proteins (Matthews, 1993).

4. Conclusions

Ebulin f and SELfd, two lectins isolated from dwarf elder fruits, have significant structural differences, revealed by their different sensitivities to pepsin. Quenching experiments revealed that Trp residues in SELfd are in a more hydrophobic environment than are those of ebulin f, which appear to be more exposed to the solvent, clearly indicating a higher degree of folding of SELfd lectin native structure than ebulin f. Binding of lactose and D-galactose to ebulin f increases protein compression, as found by quenching assays by both neutral and ionic quenchers. Sugar binding does not significantly alter the quenching pattern observed in SELfd, indicating that Trp residues are probably not directly involved in the carbohydrate binding of this lectin; pH and temperature studies indicate that SELfd shows a higher stability than ebulin f. While ebulin f opens its native structure in acidic conditions, SELfd remains stable. This might be why ebulin f is more sensitive to pepsin degradation under acidic conditions than is SELfd, as seen in a previous report. Further research remains ongoing to ascertain the importance of such pH-dependent changes for the stability of ebulin f towards pepsin degradation in the stomach and the consequent toxicity upon its interaction with the gut wall and entry into the blood.

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Figure captions

Fig 1. Fluorescence spectra of ebulin f and SELfd in absence and presence of acrylamide: (**A**) ebulin f in native conditions; (**B**) SELfd in native conditions; (**C**) ebulin f upon denaturation with 6 M Gdn.HCl; (**D**) SELfd upon denaturation with 6 M Gdn.HCl. In each panel, spectrum 1 corresponds to the protein alone and the remaining spectra (2-20) correspond to recordings in the presence of increasing concentrations of the quencher (indicated by an arrow).

Fig 2. (**A**) Stern-Volmer plots for the fluorescence quenching of ebulin f and SELfd with acrylamide. Dotted line represents the model linear prolongation of the early slope. Fixed amounts of either ebulin f or SELfd were mixed with variable concentrations of acrylamide and the intrinsic fluorescence was measured as indicated under materials and methods. (**B**) Modified Stern-Volmer plots for the fluorescence quenching of ebulin f and SELfd with acrylamide. The data used were those of Fig. 2.A.

Fig 3. Effect of temperature on intrinsic fluorescence of ebulin f and SELfd. Panel (**A**) corresponds to the fluorescence spectra of ebulin f at different temperatures, *i.e*. 4, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 °C. Panel (**C**) represents the λ_{max} values obtained for ebulin at the different temperatures assayed and the corresponding Boltzman-fit plot. Panels (**B**) and (**D**) are the equivalent graphs obtained for SELfd.

Fig 4. Effect of pH on intrinsic fluorescence of ebulin f and SELfd. (**A**) Fluorescence spectra of ebulin f under different pH conditions; (**B**) Fluorescence spectra of SELfd under different pH conditions. The λ_{max} value obtained for each treatment is also included in the legend for each panel.

Treatment	SEL_{fd}					Ebu_f				
	ΔF (%)	K_{sv1} (M^{-1})	K_{sv2} (M^{-1})	f_a (%)	K_a (M^{-1})	ΔF (%)	K_{sv1} (M^{-1})	K_{sv2} (M^{-1})	f_a (%)	K_a (M^{-1})
A	45	1.17	-----	58	2.41	72	2.43	1.92	65	11.8
B	46	1.22	-----	58	3.15	65	3.27	1.83	58	10.8
C	48	1.34	-----	61	3.02	68	2.63	1.61	56	11.4
D	82	10.7	-----	116	6.05	89	14.3	-----	111	6.62

Table 1. Main parameters obtained for the fluorescence quenching of ebulin f and SELfd with 0.5 M acrylamide in native conditions (A), in presence of 0.1 M lactose (B) or 0.1 M D-galactose (C) and upon denaturation with 6 M Gdn.HCl (D), as described under section 2.3.

Figure 1

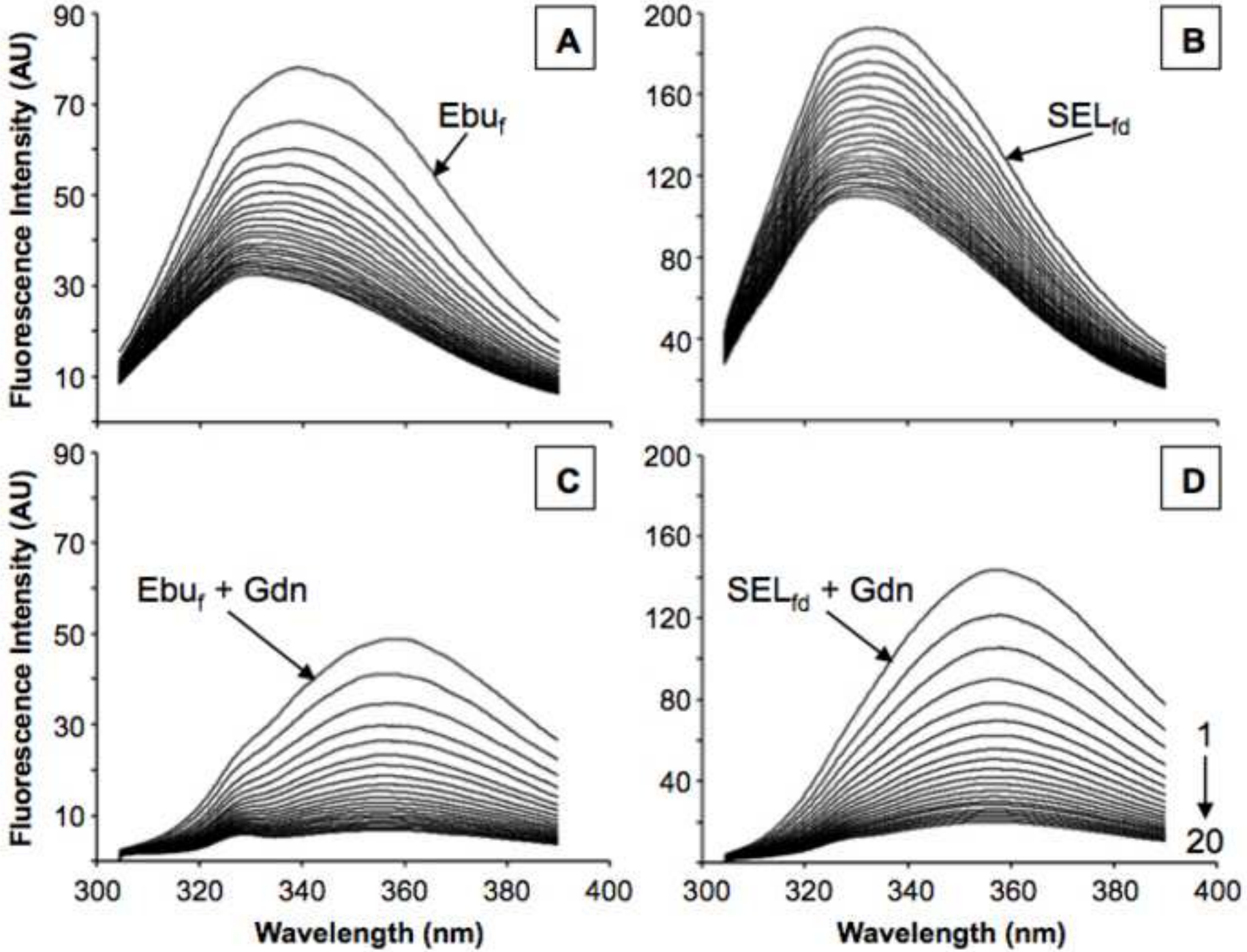


Figure 2 new

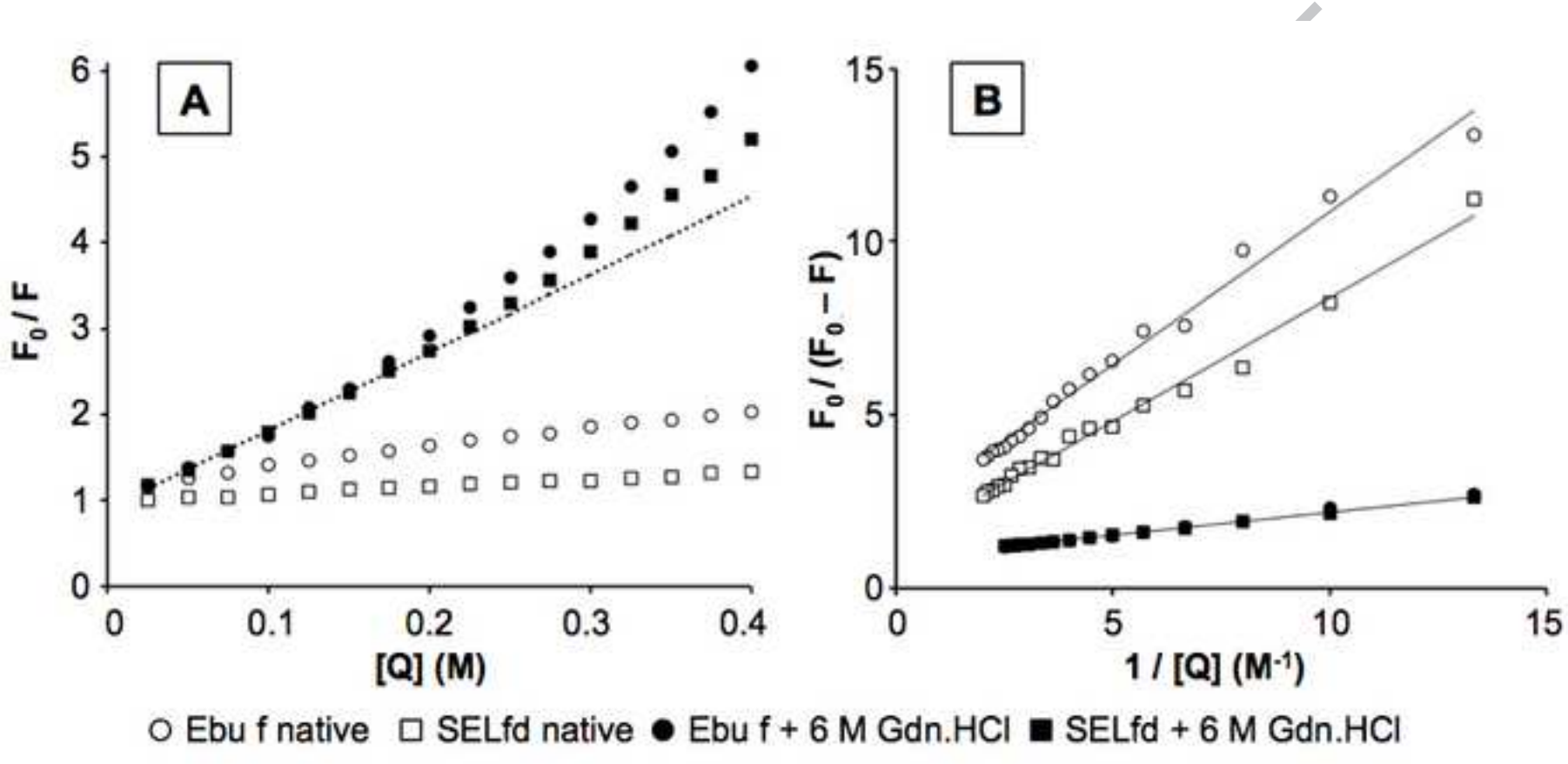


Figure 3 (old 4)

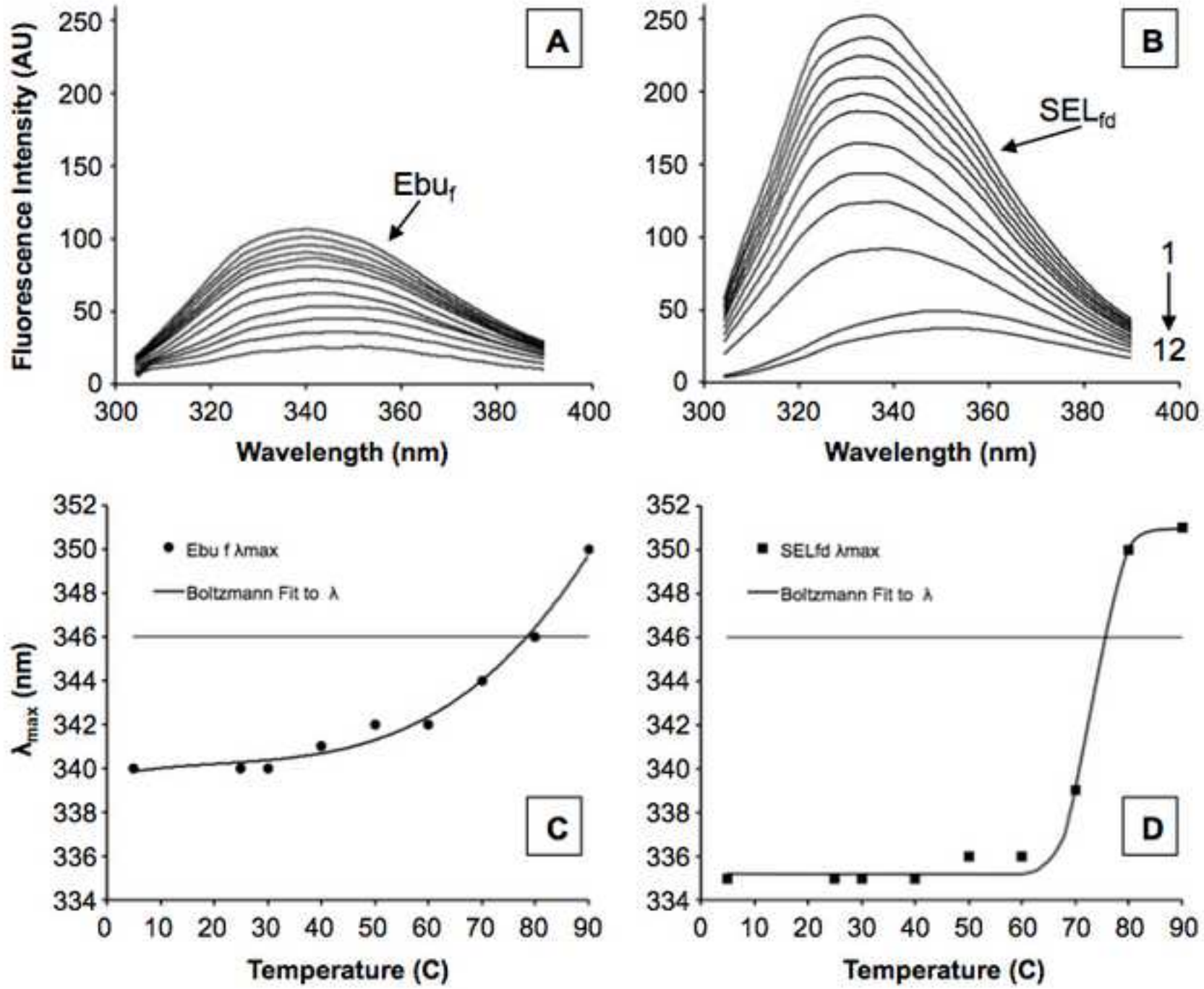
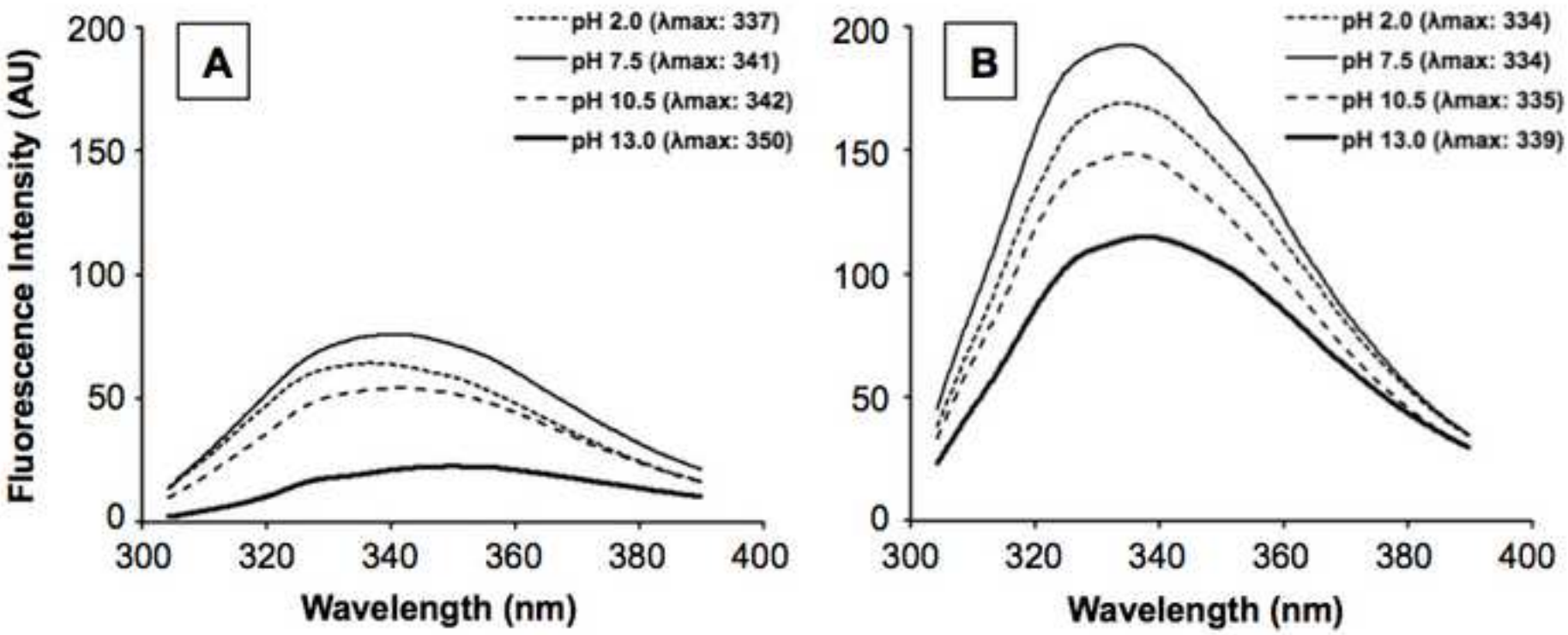


Figure 4 (old 5)



Highlights

Trp residues in SELfd are in a more hydrophobic environment than those of ebulin f.

Acidic conditions promote a more open structure of ebulin f than SELfd.

SELfd is more stable than ebulin f towards pH and temperature changes.