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The measurement of hydrophobic polypeptides in beer using the fluorochrome 1-anilino-8-naphthalenesulfonate

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Abstract

A method has been explored for assessing hydrophobic polypeptides in beer by measuring fluorescence developed upon interaction of the proteins with 1-anilino-8-naphthalenesulfonate. The method was initially applied to three separate purified proteins (bovine serum albumin, cytochrome c and catalase) and different response patterns were observed, suggesting that a simple picture would not be obtained with a complex polypeptide mixture such as beer. Furthermore substantial interference, including a high level of background fluorescence, was caused by beer components, especially in ales. Use of the method, after reducing interference by dilution or dialysis of the beer, does not appear to confer any additional value over the direct measurement of total protein in beer using Coomassie Blue-binding methods.

Keywords: Hydrophobic polypeptides; Fluorescence; 1-Anilino-8-naphthalenesulfonate

1. Introduction

It is those polypeptides with a high degree of amphipathic character that display the most foaming potential in beer (Slack & Bamforth, 1983). Through their innate hydrophobicity they have an increased tendency to move into the foam and an ability therein to interact with other hydrophobic molecules to form a stabilising framework (Simpson & Hughes, 1994).

A method for assessing the level of such polypeptides as part of a Quality Assurance strategy was suggested (Bamforth, Canterranne, Chandley, & Onishi, 1993). It involves measurement (using the staining procedure of Bradford, 1976) of the protein content of beer before and after passage through a mini-column packed with a hydrophobic interaction chromatography (HIC) matrix. By taking the difference between these two measurements, one obtains a value for the level of polypeptides possessing high hydrophobic character and which bind to the column. One potential weakness of the procedure is that it involves subtracting two relatively large numbers, leading to some unavoidable imprecision. Thus, a direct procedure for measuring these amphipathic polypeptides would be preferable.

Various methods for assessing the hydrophobicity of food proteins have been advanced (Nakai & Li-Chan, 1988). In respect of beer polypeptides, one particularly promising approach is the use of an enzyme-linked immunosorbent assay (Onishi, Proudlove, Dickie, Mills, Kauffman, & Morgan, 1999). However the necessary antibodies are not commercially available. In this paper we explore an alternative procedure based on measuring the fluorescence developed in the reaction of 1-anilino-8-naphthalenesulfonate (ANS) with proteins (Horiuchi, Fukushima, Sugimoto, & Hattori, 1978; Kato & Nakai, 1980). The fluorescence emission of ANS is critically dependent upon the environment in which it is located. Quantum yields of fluorescence are highest in non-polar environments (Turner & Brand, 1968). The reagent has been claimed to selectively react with hydrophobic regions in proteins (Daban & Guasch, 1980).

2. Materials and methods

2.1. Proteins

Samples of bovine serum albumin (BSA) Fraction V (ACROS, cat. No. 24040-0100), bovine liver catalase

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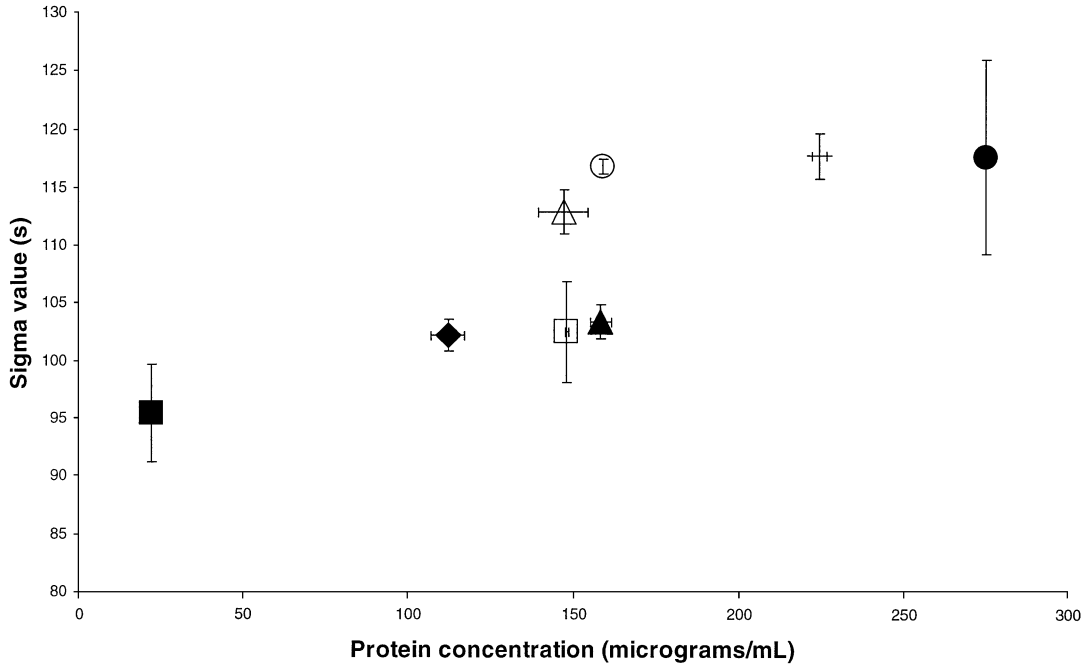

Fig. 1. The relationship of measured foam stability to total protein (Coomassie Blue binding). The beers were those investigated in Fig. 4, with the corresponding symbols.

(Sigma, cat. No. C-9322) and horse heart cytochrome c (Sigma, cat. no. C-2506) were used. Fractionation of BSA on Phenyl Sepharose was carried out according to the protocol accompanying the BRFI Foam Polypeptide Test Kit (IdQ B.V., Droevenaalssedteeg 1, NL-6708 PB Wageningen, The Netherlands).

2.2. Beers

All beers were commercial products purchased locally. Some samples were treated by either dialysis, addition of polyvinylpolypyrrolidone (PVPP, Polyclar Plus 730, ISP Technologies, Inc.) or activated carbon (Decolourising darco 20–40 mesh, ACROS). Dialysis of beers (50 ml) was against 80 volumes of deionised water for 15 h. Dialysis tubing was regenerated cellulose (cut off size 3500). PVPP (100 mg) was added to 100 ml degassed beer. After stirring for 15 min beer was clarified by centrifuging at 5000 × ***g*** for 15 min. Activated carbon was added at rates of 0.025, 0.06 or 0.1 g/ml to degassed beer. After mixing and settling (15 min) the samples were clarified by centrifuging at 5,000 × ***g*** for 15 min. Unless otherwise stated, where beers were diluted prior to determination of ANS-induced fluorescence, such dilution was by the addition of deionised water.

2.3. Measurement of protein

Protein was assessed by a Coomassie Blue binding procedure (Lewis, Krumland, & Muhleman, 1980).

2.4. Assessment of hydrophobicity by fluorescence

ANS was used according to the protocol of Hayakawa and Nakai (1985). Controls omitting ANS and protein, respectively were used. Measurements were made at ambient temperature using a SPEX Fluoromax Spectrofluorimeter with excitation wavelength 390 nm and emission wavelength 470 nm.

2.5. Assessment of foam stability

The foam stability of beers (Sigma value) was determined according to Method Beer-22A of the American Society of Brewing Chemists (1992).

2.6. Expression of results

Unless otherwise indicated, measurements represent duplicate determinations. Error bars indicate standard deviations.

3. Results and discussion

3.1. The relationship between total protein concentration and foam stability

A positive correlation between foam stability and total concentration of protein in beer was confirmed (Fig. 1). It will be noted that there is considerably more

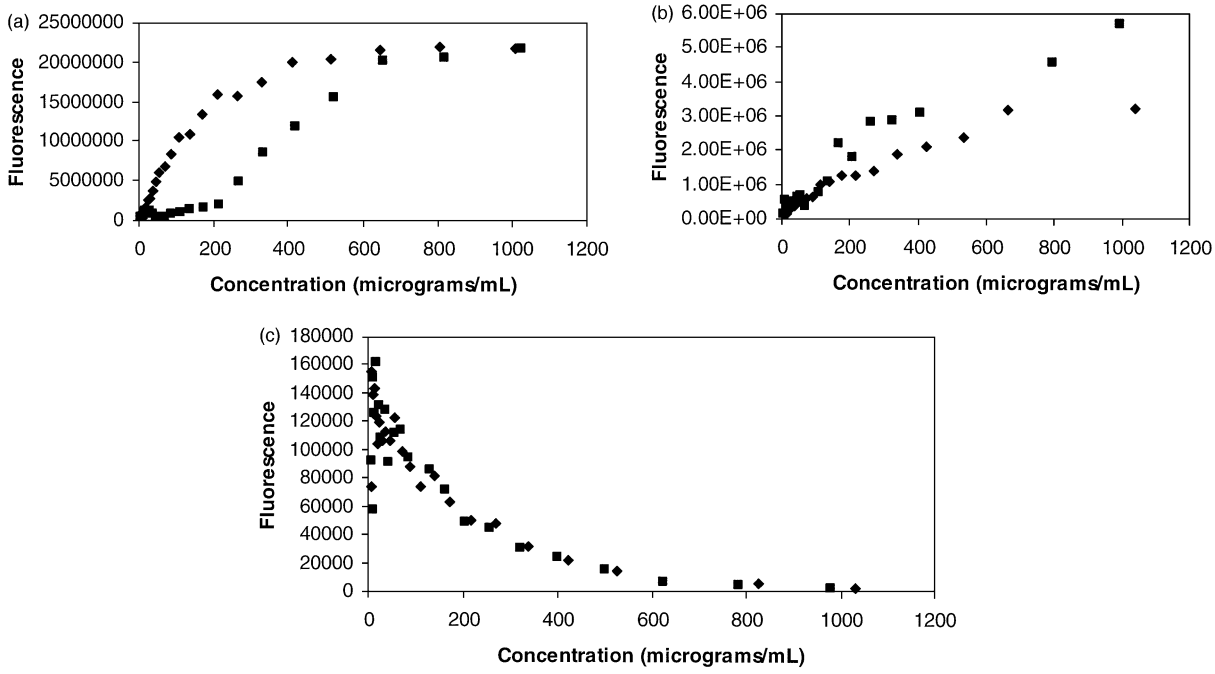

Fig. 2. The ANS-fluorescence response of purified proteins: (a) bovine serum albumin; (b) catalase; (c) cytochrome c. ◆, measurements made in 10 mM KH_2PO_4; ■, measurements made in H_2O.

data scatter for the measurement of foam stability rather than for protein concentration.

3.2. ANS studies with purified proteins

The use of ANS for assessing hydrophobic polypeptides was applied initially to three commercial protein preparations [bovine serum albumin (BSA), catalase and cytochrome c] as a forerunner to studies on beer. Although each protein showed sensibly the same response when assayed for protein by binding of Coomassie Blue (data not shown), a number of differences were observed when the proteins were assessed for fluorescence intensity upon reaction with ANS. In the case of bovine serum albumin, fluorescence was enhanced at lower concentrations of the protein when reaction was performed in the presence of 10 mM potassium dihydrogen phosphate (Fig. 2a) whereas this was not observed for catalase (Fig. 2b). Indeed, for catalase there was a quenching of fluorescence in samples containing phosphate at higher protein concentrations. Remarkably, for cytochrome c there was a distinct quenching of fluorescence at progressively higher protein concentrations, and no influence of phosphate was observed (Fig. 2c).

3.3. ANS studies with beers

The three distinct response patterns observed using BSA, catalase and cytochrome c beg the question about the extent to which the different components of a multi-

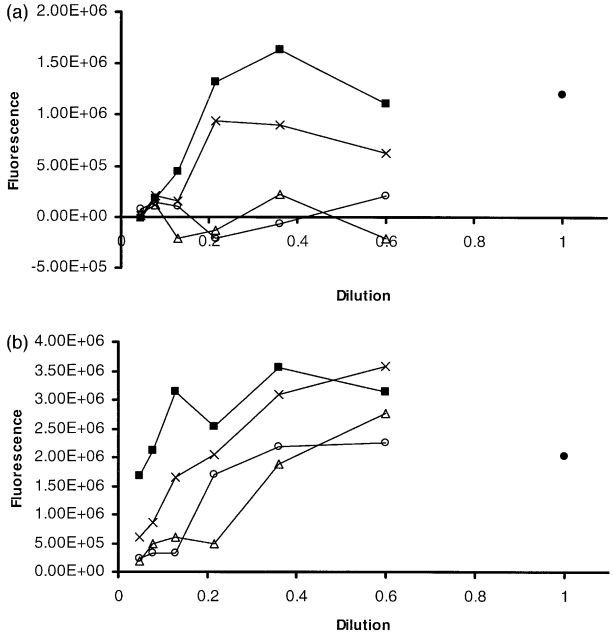

Fig. 3. The ANS-fluorescence response of pale lager beers at various dilutions: (a) Beer K; (b) Beer L. A value of 1 on the abscisssa indicates undiluted beer whilst a value of 0.25 represents 25% beer. Diluents were ■, deionised water; Δ, 10 mM KH_2PO_4; X, 5% (v/v) ethanol; ○, 5% ethanol in 10mM phosphate. Undiluted beer is represented by ●.

polypeptide system, such as beer, will respond to an agent such as ANS. Fig. 3a, b compares the fluorescence intensity at a range of dilutions of two different lager-style beers, such dilutions being made in water, phosphate or an ethanol solution of the same strength as the

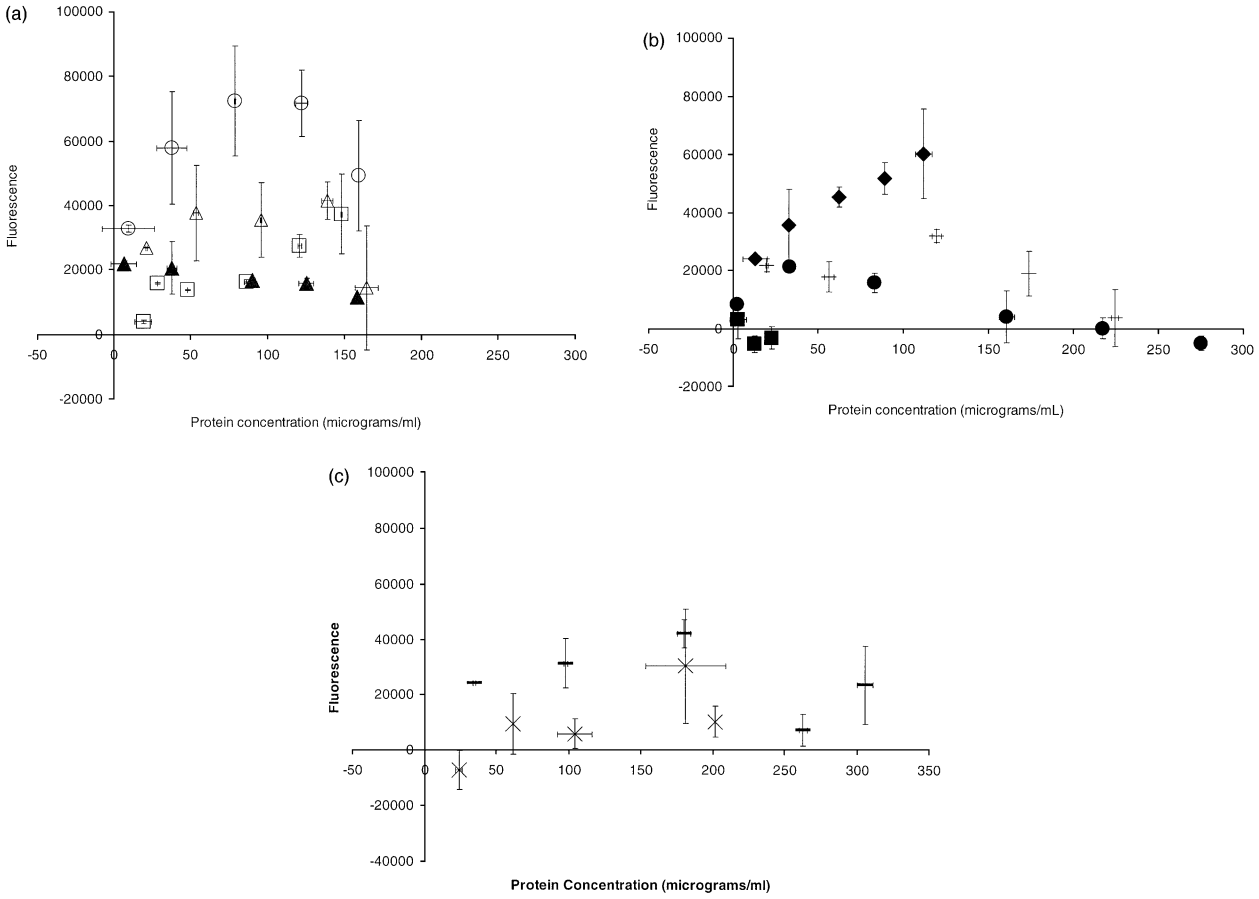

Fig. 4. The ANS-fluorescence response of a range of beers at various dilutions. Protein was measured by the Coomassie Blue dye-binding procedure. (a) A = American lager, ○; B = European lager, Δ; C = Jamaican lager,; D = American lager, ▲; (b) E = American lager, ◆; F = American lager, +; G = Mexican lager, ■; H = American lager, ●; (c) I = American ale, —; J = European ale, ×.

beer. Using phosphate and 5% ethanol as diluents led to a suppression of fluorescence in both instances.

Of even more concern was the observation (results not shown) that a number of darker beers (ales and stouts) displayed profound quenching of the fluorescence. Furthermore, even paler beers appeared to quench fluorescence unless diluted to some extent (Fig. 3).

Fig. 4a–c compares the relationship between total protein concentration (in a range derived by dilution of a series of 10 beers using deionised water) and the intensity of fluorescence obtained by reaction of such diluted beers with ANS. Various response patterns are observed. Beers A, B, C and E are pale lager beers. For each, there is essentially a linear relationship between protein concentration and fluorescence intensity, except at the very high concentrations of protein in A and B. The slope of the putative lines linking the data points for these four beers differ though, which may reflect different degrees of hydrophobicity for the polypeptides in the respective beers. If this was the case, Beer A would have more hydrophobic polypeptides than beer E, which in turn would have more hydrophobic polypeptides than beers B and C. Beer A does indeed have a higher foam stability than beer E, although it also contains a higher level of total protein (Fig. 1). Beer B also has a higher foam stability than beer E, and again it contains a higher total protein concentration. The foam stabilities of beers A and B are very similar, in keeping with their similar total protein concentrations. There is no indication that the higher fluorescence:protein ratio in beer A leads to greater foam power. High degrees of fluorescence suppression were observed for the other beers studied, which were darker products (either ales or stouts).

All beers displayed strong background fluorescence in controls from which ANS was omitted (examples are shown in Figs. 5a–d). At higher protein concentrations there was inherent quenching of this fluorescence. In most cases, the amount of fluorescence emission registered in response to the presence of ANS is somewhat less than that displayed as background, which contributes to the relatively large errors associated with ANS-fluorescence values (Fig. 4). This background fluorescence can be reduced considerably by dialysis (Fig. 6), but such a step is not commensurate with a simple, rapid and reliable protocol for use in routine quality assurance operations. Dialysis of the beers also led to some diminution in quenching, consistent with a contribution from a low molecular weight interferent

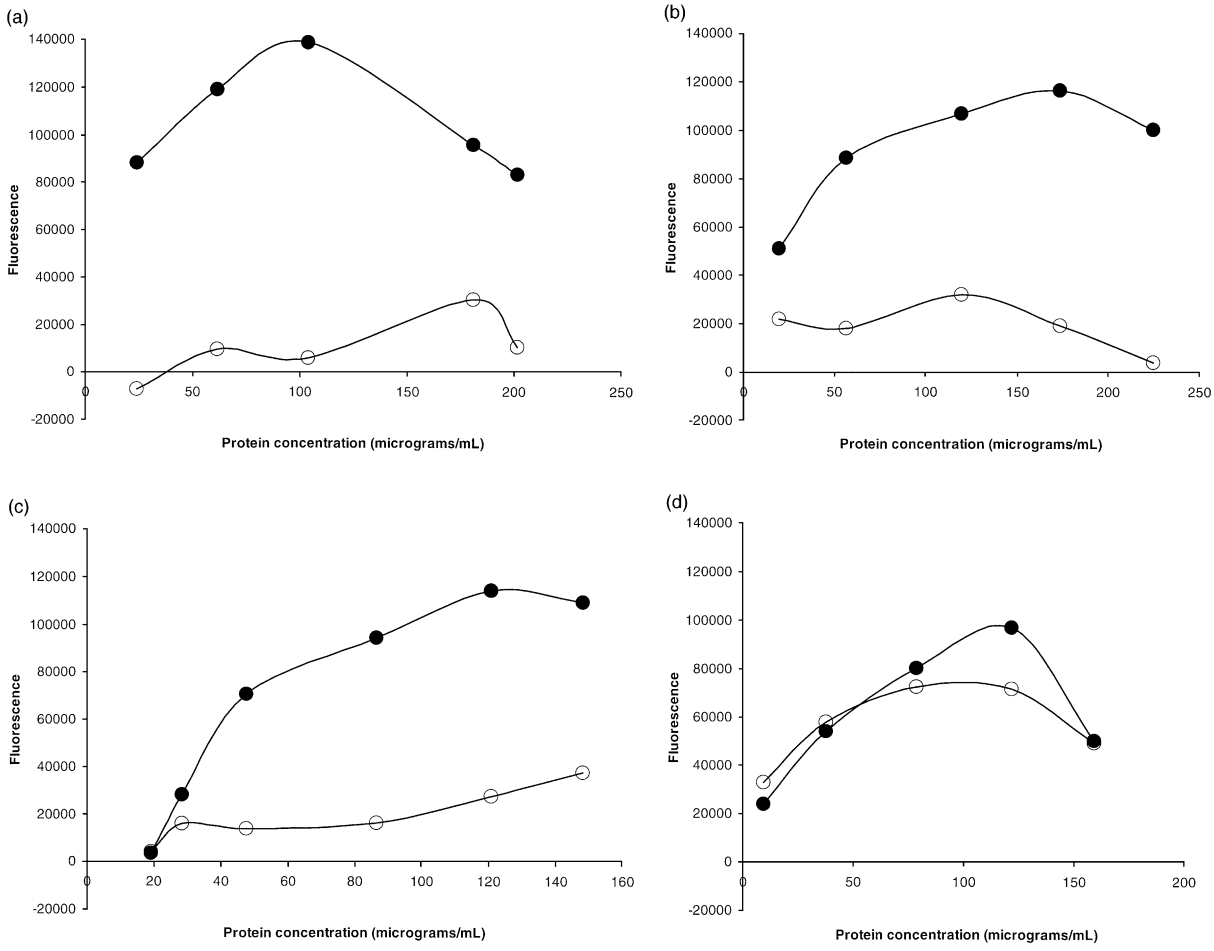

Fig. 5. The inherent fluorescence concentration of beers at various dilutions (●) compared with the fluorescence obtained upon reaction with ANS (i.e. background fluorescence subtracted) (○). (a) to (d) represent different beers. Dilutions were made using deionised water.

(Fig. 6). Treatment of the beers with activated carbon to remove coloured materials removed a significant proportion of the beer protein, but had only a small impact on the background fluorescence and on the quenching phenomenon (data not shown). PVPP had little impact in terms of relieving the quenching or background fluorescence, indicating that polyphenols are not responsible for this interference (data not shown). It would appear that most of the background fluorescence in beers, as well as the inherent quenching of fluorescence (both background and that caused by the ANS interaction) is due to relatively high molecular weight materials which are for the most part not removable by activated carbon or PVPP. They may be proteins per se or materials binding to proteins.

The considerable interference in the fluorescence reaction displayed by many beers does not augur well for the ANS-fluorescence method as a routine procedure for assessing the level of hydrophobic polypeptides in beer. However, as there seems to be less quenching of fluorescence at low protein concentrations for most products, we have examined the worth of the ANS-fluorescence procedure for assessing the level of hydrophobic polypeptides in highly diluted beers, comparing it with measured foam stability. Fig. 7 shows that a reasonable correlation is observed between foam stability and ANS-fluorescence intensity measured in beer samples diluted to 1 and 2%. However, beers displaying

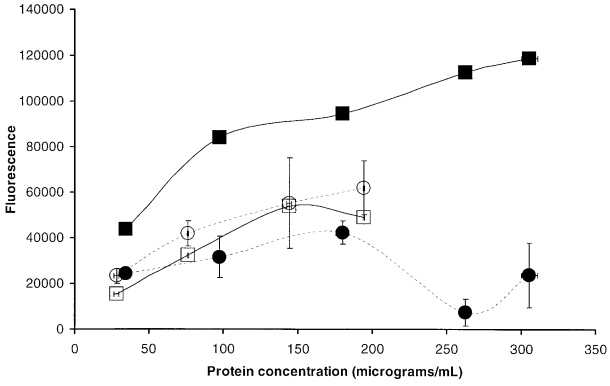

Fig. 6. The effect of dialysis on background and ANS-induced fluorescence. The beer used was I in Fig. 4. ■, Background fluorescence; □, background fluorescence after dialysis; ●, ANS fluorescence (less background fluorescence); ○, ANS fluorescence (less background fluorescence) after dialysis.

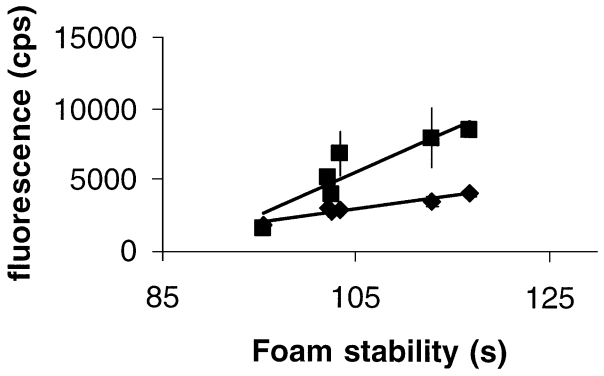

Fig. 7. The relationship between foam stability and ANS-fluorescence measured in beers diluted to 1% (◆) and 2% (■).

high background fluorescence eliminate the correlation. When taking all beers into consideration we could not demonstrate a superior correlation to that observed between foam stability and total protein (in undiluted beer) as quantified by dye binding (Fig. 1). Even in this case the correlation is weak, though the errors associated with the measurement of foam stability are considerable.

3.4. Critique of ANS-fluorescence as a technique for assessing amphipathic polypeptides in beer

From the studies reported in this paper, there seems to be neither practical nor functional advantage from the use of ANS-fluorescence response as a tool for quantifying beer foaming proteins. Clearly, interferences are encountered which render the interpretation of data difficult and confusing. Furthermore, the assumption has been made that ANS is indeed an agent that specifically binds to hydrophobic regions in proteins. Whilst this is the supposition of Nakai and coworkers (Hayakawa & Nakai, 1985; Kato & Nakai, 1980) and was indeed the conclusion of detailed studies on apomyoglobin (Stryer, 1968), it has been suggested by Slavik (1982) that the reagent may react with proteins in ways other than with hydrophobic groups alone. This is one explanation for our finding that when BSA was fractionated on the column used in the HIC test, most of the ANS-dependent fluorescence was associated with the fraction that does not bind to the column. The ratio of fluorescence intensity to total protein concentration was similar in both 'hydrophilic' and 'hydrophobic' fractions. However, it is also possible that a substantial amount of polypeptide with hydrophobic character emerges in the so-called 'hydrophilic' fraction and that the ANS reagent is detecting at least as much hydrophobic character in proteins that do not bind to the HIC column as in those which do. The binding capacity of the gel exceeds 10 mg protein per ml of gel and in no instance was more than 1 mg protein applied to the column, i.e. the column was not overloaded. In fact, a substantial amount of protein was not recovered from the column in this type of experiment. These observations may simply indicate the high sensitivity of ANS as compared to the gel as a means for detecting hydrophobicity. That is, weak hydrophobicity insufficient to allow binding to Phenyl Sepharose may nonetheless be detected fluorimetrically. Even so, very similar results were observed when the more hydrophobic Octyl Sepharose was used in place of Phenyl Sepharose.

A range of factors impact on the quality and stability of beer foam, of which the content of protein in the beer is just one (Bamforth, 1985). Thus, it might not be expected that a particularly precise relationship would be observed between a measurement of protein and foam stability. Furthermore, the techniques by which foam stability itself are assessed introduce their own vagaries into the interpretation. These matters being considered, we can probably hope for no better correlation than that observed in Fig. 1, in which total protein is assessed by a dye-binding method (Siebert & Knudson, 1989). This is not to suggest that the amphipathic character of protein is irrelevant to foaming: the clear evidence is that it is (Hughes et al, 1998; Onishi & Proudlove, 1994). However we must recognise that all proteins will have hydrophobic character to a greater or lesser degree. For many it is hidden within the molecule, but is increased by exposure of the inner regions upon denaturation (Nakai, 1983). Coomassie Blue-binding procedures are simple and straightforward and, in the context of the myriad of factors influencing foam, are probably as reliable a means as any other for indicating whether or not a beer is likely to be deficient in foam protein. Those convinced of a need for a more specific assessment technique might consider the use of the immunological tests incorporating antibodies raised against hydrophobic polypeptides (Onishi et al., 1999). The ANS procedure, however, should not be ignored for the value it may have in probing the properties of isolated polypeptides. Furthermore, a more detailed investigation of the fluorescence inherent in beers is warranted.

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