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Structure–function relationships of cowpea (*Vigna unguiculata*) globulin isolate: influence of pH and NaCl on physicochemical and functional properties

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Physicochemical and functional properties of cowpea globulin isolate were determined as a function of pH and NaCl concentrations. Protein solubility (PS) increased with increasing pH from 3 to 8, while at low pH, PS decreased with increasing ionic strength. At low pH and all ionic strengths, the protein isolate was extensively coagulated by heat, while aromatic hydrophobicity, fluorescence intensity and emulsifying activity index were all higher than results obtained at high pH. Emulsion stability was lowest at low pH and ionic strength. Foaming capacity increased with increasing pH and ionic strength. Foam stability was affected more by pH changes than by ionic strength. The results were discussed on the basis of protein–protein and protein–solvent interactions, as affected by the balance between electrostatic repulsions and hydrophobic interactions.

INTRODUCTION

The use of plant proteins in the formulation of new food products or as a replacement for more expensive animal proteins in conventional foods has been the focus of various research efforts. In order to develop plant proteins for use as ingredients in the food industry, there is the need to determine the physicochemical and functional properties of these proteins. Cowpea (*Vigna unguiculata*) is an important food crop in tropical and subtropical regions, especially West Africa (Khan *et al.*, 1980). Cowpea protein isolates have been studied and shown to possess some desirable functional properties (Sefa-Dedeh & Yiadom-Farkye, 1988; Aluko & Yada, 1993). An important consideration in the study of protein isolate functionalities is their response to the environment, of which pH and ionic strength are the most important (Myers, 1988).

Molecular forces such as hydrophobic and electrostatic interactions can be manipulated by pH and ionic strength to produce proteins with varying structural conformations and hence functional properties. The effects of pH and/or ionic strength on solubility, emulsifying and foaming properties of soy flour, sesame seed, soy protein isolates, β-lactoglobulin and bovine serum albumin have been reported (Shen, 1976; Kamat *et al.*, 1978; McWatters & Holmes, 1979; Shimizu *et al.*, 1985; Mitchell, 1986; Prakash, 1986). Generally, it was shown that protein solubility was low at low pH in the presence of salt, whereas the emulsifying and foaming properties did not follow a particular pattern but varied according to the source of protein (Shen, 1976; Kamat *et al.*, 1978; McWatters & Holmes, 1979; Shimizu *et al.*, 1985; Mitchell, 1986; Prakash, 1986).

The present work was undertaken to determine the physicochemical and functional properties of cowpea globulin isolate as a function of pH and ionic strength.

MATERIALS

Cowpea seeds (var. SAMPEA-7, commonly called IAR-48) were obtained from the Institute for Agricultural Research, Ahmadu Bello University, Zaria, Nigeria. The seeds were harvested in November 1991 and contained 7·6% moisture and 21·7% crude protein (% Kjeldahl N × 5·7).

METHODS

Preparation of cowpea flour

Seeds were ground into flour in a Moulinex 980 coffee grinder (Moulinex, France) at full speed in 5 cycles of 1 min each with 5-min rest intervals to prevent excessive heating. The flour was then defatted with acetone at 4°C

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for 24 h with four changes of acetone. The acetone was decanted and the flour air-dried. The dried flour was then passed through a 60-mesh sieve and used for subsequent protein extraction.

Preparation of globulin isolate

Globulins were extracted from the sieved flour according to the method of Khan *et al.* (1980) with some modifications. Extraction was carried out for 1 h using 0·25 M Tris-HCl buffer (pH 8·0) containing 0·5M NaCl at a ratio of 1:20 (flour:solvent). The slurry was then centrifuged at 20000*g* for 30 min at 4°C and the supernatant filtered through Whatman number 1 paper. The filtrate was dialyzed against distilled–deionized water for 48–72 h at 4°C with 8–10 changes of water, to precipitate the salt-soluble proteins. The precipitated proteins were recovered by centrifugation at 20000*g* for 30 min, followed by washing once with distilled–deionized water and centrifuged again. The resultant precipitate was then freeze-dried, labelled as globulin isolate and stored at 4°C.

Protein solubility

Solubility of the isolate was determined according to the method of Aluko and Yada (1993) with modifications. Sample dispersions (0·1% w/v) were prepared in 0·01 M Na_2HPO_4 adjusted to pH 3–8 with 0·1M HCl and contained either 0·5, 1, 1·5 or 2 M NaCl. For total soluble protein content (control), the isolate was dispersed in 0.1M NaOH. The samples and control were then mixed on a magnetic stirrer (Fisher Scientific, Fairlawn, NJ, USA) at 70% of the maximum speed setting, followed by centrifugation at 20000*g* for 30min. The supernatant was filtered through Whatman number 1 paper and the amount of soluble protein in the filtrate determined by the method of Hartree (1972). Percent protein solubility (PS) was calculated as follows:

$$PS(\%) = \frac{\text{Protein content of sample} \times 100}{\text{Protein content of control}}$$

Preparation of protein stock solutions

Sample dispersions (0·5% w/v) were made in 0·01 M Na_2HPO_4 (adjusted to pH 3–8 with 0·1M HCl) containing either 0·5, 1, 1·5 or 2 M NaCl. The dispersions were mixed on a magnetic stirrer for 30 min and centrifuged at 4°C and 20000*g* for 30 min. The supernatant was filtered through Whatman number 1 paper and the amount of protein in the filtrate determined according to the method of Hartree (1972). The protein stock solutions were diluted to the required concentration for determination of physicochemical and functional properties.

Native-PAGE

SDS–PAGE could not be conducted since the proteins precipitated on addition of SDS containing buffer, probably as a result of the high level of salt in the protein preparation. No protein bands were evident when the supernatant was run, while the precipitate showed poor resolution even after addition of mercaptoethanol and heating (5 min in a boiling water bath). Therefore, native-PAGE was performed as described by Marcone and Yada (1991) using the PhastSystem Separation, Control and Development unit (Pharmacia, LKB).

UV absorption spectrophotometry

Absorption spectra of 0·01% (w/v) protein solutions were recorded from 250 to 350 nm at 25°C using quartz cuvettes (1 cm pathlength) on a UV-visible recording spectrophotometer (Model UV-260, Shimadzu Corporation, Kyoto, Japan). The maximum absorbance (Amax) was recorded for each sample.

Fluorescence emission spectrophotometry

The protein stock solution was diluted to 0·002% (w/v) and emission spectra recorded at 25°C using a 1 cm pathlength cuvette on a Shimadzu spectrofluorophotometer (Model RF-540, Shimadzu Corporation). Protein samples were excited at 280 nm and emission recorded from 300 to 400 nm. Emissions of the buffer blanks were subtracted from those of the respective samples, and the maximum fluorescence intensity (FI) recorded.

Aromatic hydrophobicity

The stock protein solutions were each diluted to give protein concentrations ranging from 0·00125 to 0·01% (w/v). Aromatic hydrophobicity (ARH) was then determined using 1-anilino-8-naphthalene sulfonate (ANS) as a probe, according to the method of Hayakawa and Nakai (1985).

Functional properties

Protein stock solutions were diluted to 0·1% (w/v) concentration for measurement of functional properties. Emulsifying activity index (EAI, m^2/g), emulsion stability (ES, %), foaming capacity (FC, ml), and foam stability (FS, %) were determined as described by Aluko and Yada (1993), except that the FC values were reported as volume of foam (ml). Heat coagulability (HC, %) was determined according to the method of Voutsinas *et al.* (1983).

Statistical analysis

Three-dimensional plots of physicochemical and functional properties as a function of pH and NaCl were produced using the SAS/GRAPH (1990) package. Each analysis was done in duplicate with two determinations for each replicate. Data were first analyzed by the G3GRID procedure using the 'SPLINE' option to

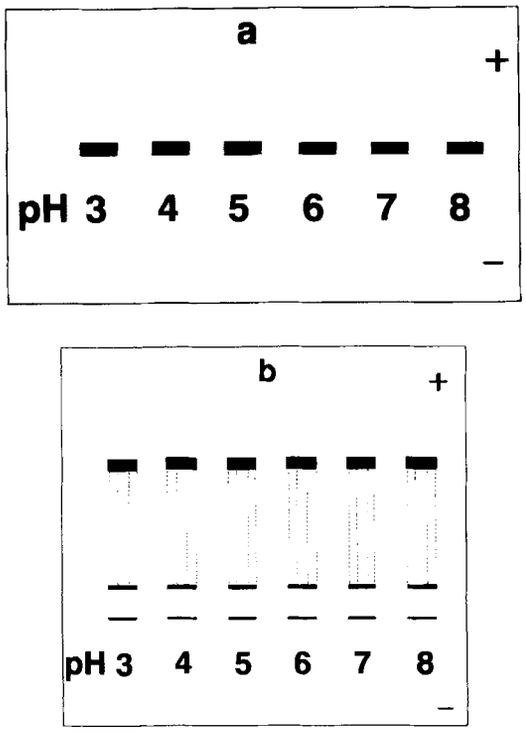

Fig. 1. Illustrations of the native gel electrophoresis patterns obtained for cowpea globulin isolate containing (a) 0·5 M and (b) 1·5 M NaCl. Similar gel patterns were seen for 1 M as compared to 0·5 M NaCl and 2 M as compared to 1·5 M NaCl.

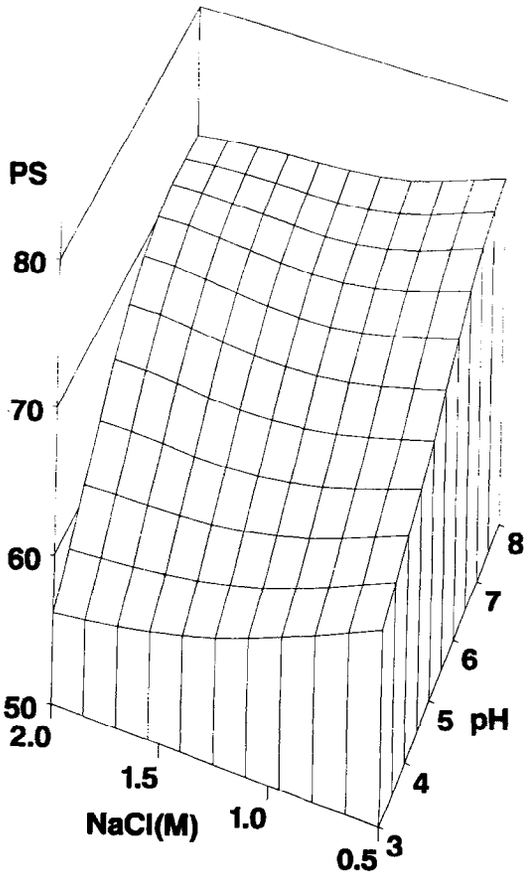

Fig. 2. Protein solubility (PS, %) plotted as a function of pH and NaCl concentration. Response surface was generated on the basis of duplicate samples (with 2 determinations each) for defined pH (3, 4, 5, 6, 7 and 8) and ionic strengths (0·5, 1, 1·5 and 2 M).

ensure smooth surface of the subsequent plots. Data from the G3GRID procedure were then used as the input data for the G3D procedure which produced the three-dimensional plots.

RESULTS AND DISCUSSION

Native-PAGE

Schematic representations of the gel patterns from native-PAGE are shown in Figs 1a and b. One major band was commonly observed at all pH and NaCl levels. However at 1·5 and 2 M NaCl concentrations, two additional smaller protein bands were observed close to the cathode (Fig. 1b) and were thought to be of higher molecular weight, although charge and shape may have been factors. Moreover the gels for 1·5 and 2 M NaCl concentrations showed protein band tailing (indicated in Fig. 1b as broken lines), which is indicative of low PS and/or high salt concentration (Pharmacia LKB, PhastSystem Operation Manual). This suggests that at higher ionic strengths (1·5 and 2 M NaCl), there is increased protein association, resulting in formation of high-molecular-weight aggregates. Such molecular aggregation could be attributed to increased association of the salt ions with the protein charges to reduce intermolecular repulsion (Kinsella *et al.*, 1985). Similar gel patterns were obtained for a cowpea isoelectric protein isolate under identical experimental conditions, i.e. 0·01 M Na_2HPO_4 pH 3–8 containing either 0·5, 1, 1·5 or 2 M NaCl (Aluko & Yada, 1994, unpublished data).

Physicochemical properties

PS as function of pH and NaCl is shown in Fig. 2. At low pH (3–4), solubility of the globulin isolate decreased with increasing ionic strength. As the pH increased, the effect of NaCl diminished. PS at higher pH values were generally similar irrespective of the ionic strength. Generally, PS increased with increasing pH for each NaCl concentration. This result can be explained on the basis of protein–protein and protein–solvent interactions. At low pH, all the carboxyl groups are protonated and the protein has a net positive charge. Electrostatic repulsive forces between the positively charged proteins help to keep them apart and increase protein–solvent interactions (Kinsella *et al.*, 1985). Addition of NaCl causes negatively charged chloride ions to interact with the positively charged proteins, thereby decreasing electrostatic repulsions and enhancing hydrophobic interactions. The increase in hydrophobic interactions would result in a higher tendency for the protein to form insoluble aggregates, thus decreasing solubility. However, at high pH values, the increased net negative charge on the protein, coupled with the salting-in effect of NaCl on the hydrophobic interactions (Kinsella *et al.*, 1985), dissociates the protein aggregates, and solubility increases. Similar results have been reported for soy flour (McWatters &

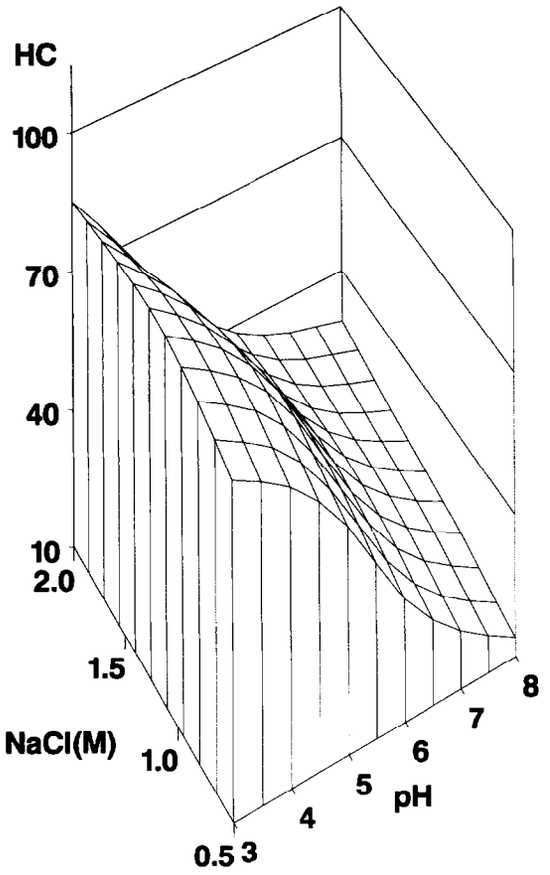

Fig. 3. Heat coagulability (HC, %) plotted as a function of pH and NaCl concentration. Response surface was generated on the basis of duplicate samples (with 2 determinations each) for defined pH (3, 4, 5, 6, 7 and 8) and ionic strengths (0·5, 1, 1·5 and 2 M).

Holmes, 1979), sesame seed proteins (Prakash, 1986) and cowpea isoelectric protein isolate (Aluko & Yada, 1994, unpublished data).

Figure 3 shows the effects of pH and ionic strength on heat coagulability of the globulin isolate. Generally, the globulin isolate was extensively coagulated at pH 3 but decreased gradually as the pH increased, regardless of the ionic strength, a result similar to that reported by Aluko and Yada (1994, unpublished data) for cowpea isoelectric protein isolate. The HC response surface shows an inverse pattern to that of PS, suggesting that the lower the solubility of the protein the higher its susceptibility to coagulation by heat. This is expected since the decreased electrostatic repulsions operating at low pH in the presence of salt (Kinsella *et al.*, 1985) would enhance protein association, especially under the influence of heat which ultimately leads to coagulation.

The response surfaces for A_{max}, FI and ARH are shown in Figs 4a, b, and c, respectively. A combination of UV-absorption data and fluorescence emission spectra has been suggested as a useful tool towards probing the exposure of aromatic residues in proteins, and hence of surface (aromatic) hydrophobicity (Jackman & Yada, 1989). The present results show a general similarity between the response surfaces of FI and ARH, but not A_{max}. Therefore, it is reasonable to suggest that both FI and ARH may be more useful than A_{max} in furnishing information about the exposure of aromatic groups on the surface of cowpea globulin isolate. Generally, similar results have been reported for a cowpea isoelectric protein isolate (Aluko & Yada, 1994, unpublished data).

The high ARH and FI obtained at low pH in the presence of salt provides further evidence that masking of protein charge could lead to an increased hydrophobic character of the protein (Kinsella *et al.*, 1985). In the present work, no apparent difference between the four levels of ionic strength with respect to interaction of the ANS probe with the protein was observed, since at any given pH the ARH was similar at all NaCl concentrations. The quantum yield of the ANS probe has been documented to be insensitive to pH in the range 2–8 (Gibrat & Grignon, 1982). Therefore, it appears that the effects of pH on ARH are due mainly to protein–probe interaction changes with different pH and not pH-dependent fluorescence intensity of the probe.

Functional properties

The EAI as a function of pH and sodium chloride concentrations are shown in Fig. 5a. The response surface showed a high EAI at low pH which decreased progressively with increasing pH. It was stated earlier that there was increased protein–protein (hydrophobic) interaction at low pH in the presence of salt, a situation which could favor emulsion formation by increasing the rheological properties of the interfacial protein films which encapsulate the oil droplets. An increased rheological strength of the protein films could reduce mechanical deformation and desorption of the interfacial proteins to give more emulsified droplets (Halling, 1981). Formation of emulsions have been shown in past reports to be favored by high surface hydrophobicity (Kato & Nakai, 1980; Li-Chan *et al.*, 1984; Nakai *et al.*, 1986). The low EAI at high pH may be due to the increased repulsive forces resulting in poor rheological properties of the interfacial film.

ES was low at both low pH and NaCl concentrations but increased with a simultaneous increase in both pH and ionic strength (Fig. 5b). The increase in ES may have been achieved through formation of charged layers around the fat globules resulting in mutual repulsion and/or by forming a hydrated layer around the interfacial material, factors which lower interfacial energy and retard droplet coalescence (Kinsella *et al.*, 1985).

Figure 6a shows the FC of the protein isolate as a function of pH and ionic strength. The response surface shows a general increase in FC as the pH and ionic strength increased. This latter result may be due to the fact that foam formation is favored by protein flexibility and limited intermolecular cohesion (Kinsella *et al.*, 1985). At high pH, the decrease in attractive hydrophobic forces as a result of the increased net charge on the protein could increase flexibility and enable the protein to diffuse more rapidly to the air–water interface to encapsulate air particles. Also, increasing salt concentration increases the propensity for adhesion

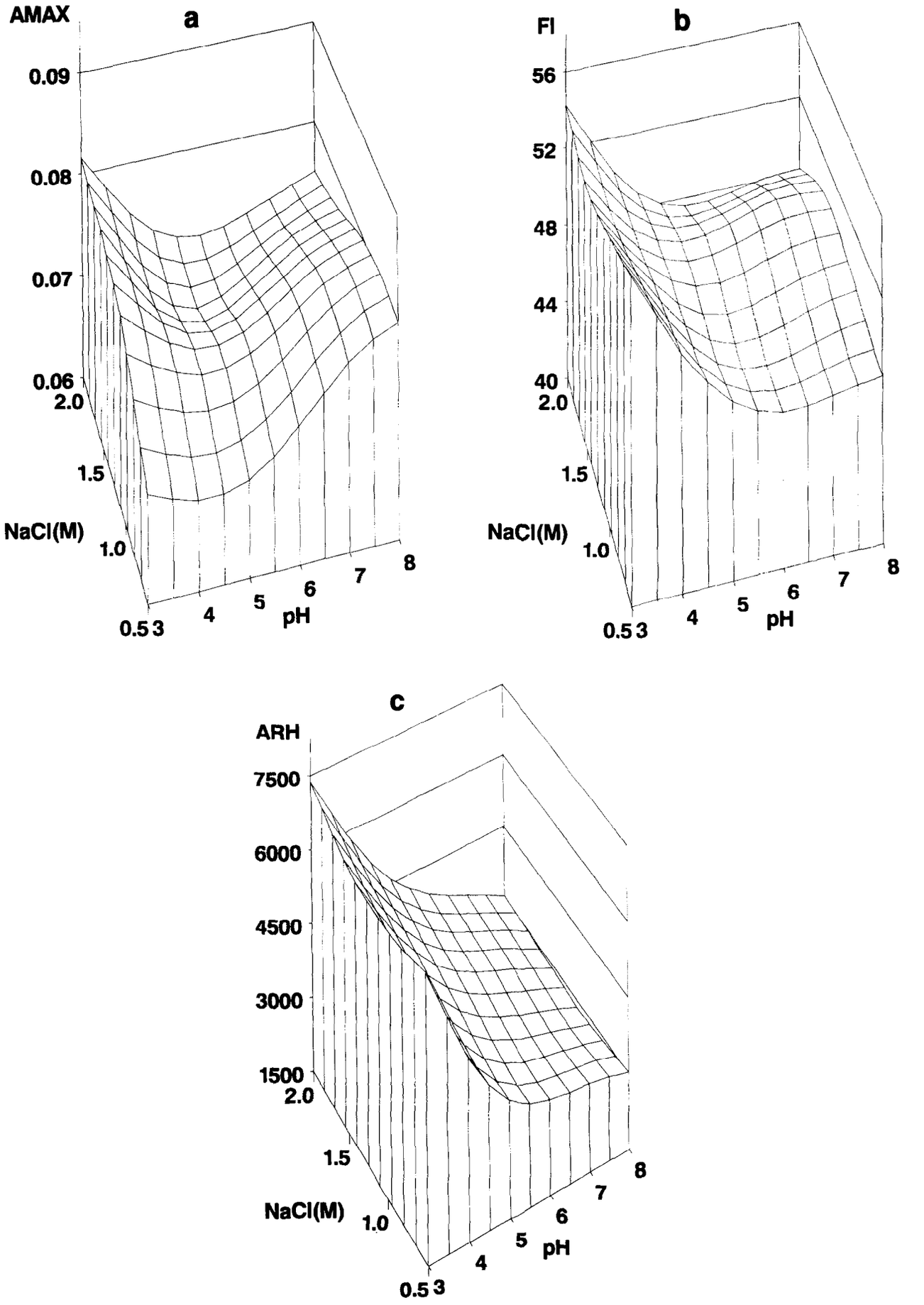

Fig. 4. (a) UV-absorption maxima (A_{max}), (b) fluorescence intensity (FI) and (c) aromatic hydrophobicity (ARH), each plotted as a function of pH and NaCl concentration. Response surfaces were generated on the basis of duplicate samples (with 2 determinations each) for defined pH (3, 4, 5, 6, 7 and 8) and ionic strengths (0·5, 1, 1·5 and 2 M).

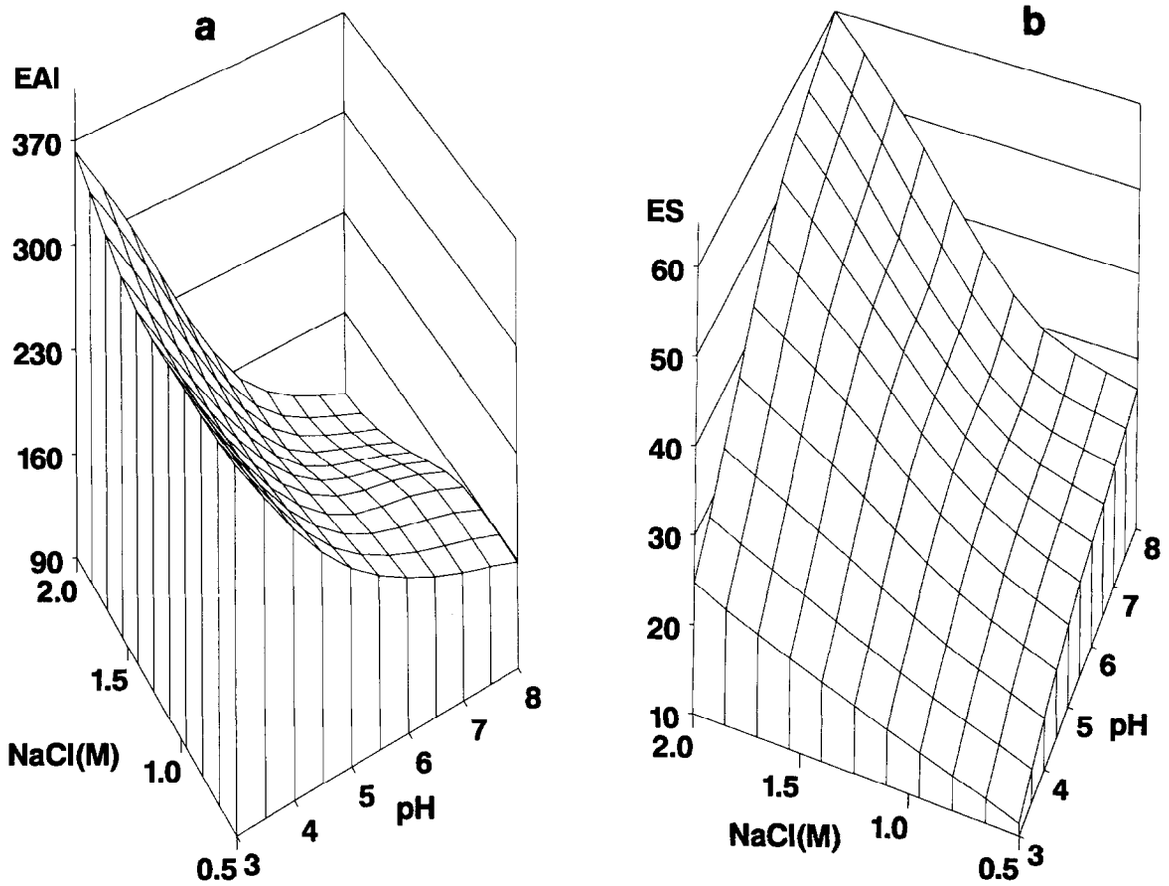

Fig. 5. (a) Emulsifying activity index (EAI, m^2/g) and (b) emulsion stability (ES, %), each plotted as a function of pH and NaCl concentration. Response surfaces were generated on the basis of duplicate samples (with 2 determinations each) for defined pH (3, 4, 5, 6, 7 and 8) and ionic strengths (0·5, 1, 1·5 and 2 M).

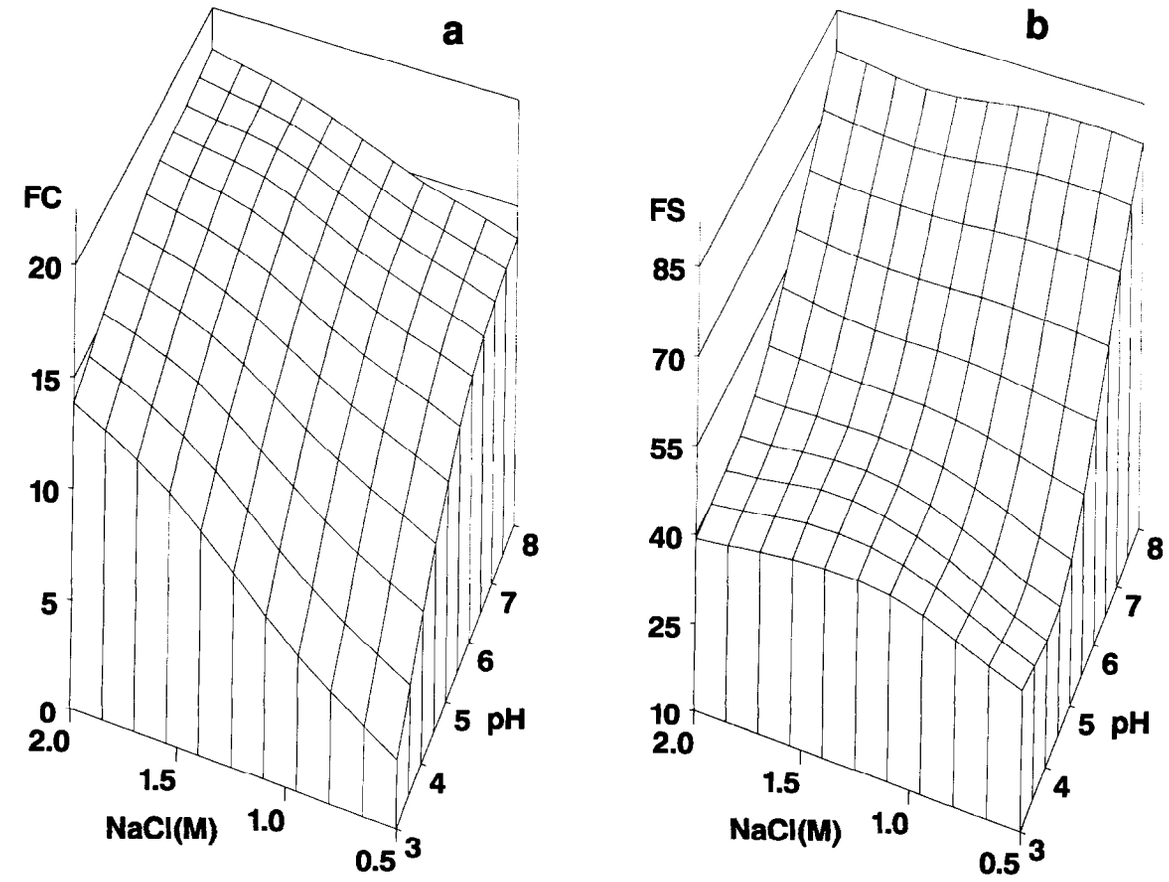

Fig. 6. (a) Foaming capacity (FC, ml) and (b) foam stability (FS, %), each plotted as a function of pH and NaCl concentration. Response surfaces were generated on the basis of duplicate samples (with 2 determinations each) for defined pH (3, 4, 5, 6, 7 and 8) and ionic strengths (0·5, 1, 1·5 and 2 M).

between the protein molecules and results in the formation of interfacial proteins with stronger rheological properties to maintain foam integrity during mechanical whipping. Similar results were obtained for soybean proteins (Cherry & McWatters, 1981) and cowpea isoelectric protein isolate (Aluko & Yada, 1994, unpublished data).

The effects of pH and ionic strength on FS are shown in Fig. 6b, indicating that the foams were better stabilized at higher pH, irrespective of the ionic strength. In foams, the ability to hold water in the protein film surrounding the air particle and presence of electrostatic repulsions have been suggested to be important for stability (Kinsella *et al.*, 1985; Myers, 1988). The present result is consistent with these suggestions, since the increased FS at higher pH may have been due to a decreased tendency towards foam particle coalescence as a result of the higher net negative charge on the protein. There was no observable difference between the levels of NaCl, as changes in FS seemed to be more dependent on pH.

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