Proteins from *Crambe abyssinica* Oilseed. II. Biochemical and Functional Properties

E. Massoura*, J.M. Vereijken, P. Kolster, and J.T.P. Derksen

Agrotechnological Research Institute (ATO-DLO), 6700 AA Wageningen, The Netherlands

ABSTRACT: Little information is available concerning the properties of proteins that constitute a major component of the seed of *Crambe abyssinica*. Therefore, a method was developed to isolate these proteins. This procedure resulted in two fractions, an isoelectric precipitate (Fraction 1) and a retentate after ultrafiltration (Fraction 2). Biochemical and functional properties of both fractions were studied. Gel permeation chromatography revealed that high-molecular-weight proteins (>669,000 Da) are present only in Fraction 1, whereas Fraction 2 consists of proteins with lower molecular weights (<200,000 Da). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, in the presence of mercaptoethanol, showed that both fractions consist of proteins that range mainly from 40,000 to less than 14,400 Da. Fraction 1 was highly soluble only at acid and alkaline pH values, while the solubility of Fraction 2 remained high (>80%) over the whole pH range tested. Addition of NaCl did not have any profound effect on the solubility of Fraction 2, but it increased significantly that of Fraction 1 in the isoelectric range. Foaming properties of Fraction 1 were better than those of chicken egg white only at pH 9, whereas those of Fraction 2 were superior at almost every pH value studied. Addition of NaCl improved significantly the foaming properties of Fraction 1 at all pH values tested but did not have a profound effect on the foaming properties of Fraction 2. Both fractions had good emulsifying properties only at alkaline pH values.

JAOCS 75, 329–335 (1998).

KEY WORDS: *Crambe abyssinica,* functional properties, molecular weight, proteins.

Crambe abyssinica is a promising new crop for the production of oil for industrial applications. For the economics of processing this crop for oil production, applications of the by-product meal as a whole or of its major components, such as the protein fraction, could be of decisive importance. Most research has been concerned with the potential use of *Crambe* meal as animal feed (1–6). The suitability of the proteins for both food and nonfood applications with a higher added value is not yet known.

In the preceding paper (7), an isolation procedure is described for proteins from dehulled, defatted *Crambe* meal. After alkaline extraction, about half of the proteins present in the extract are recovered by isoelectric precipitation. This isoelectric precipitate constitutes one protein fraction. The remaining solution is subjected to ultrafiltration. The resulting retentate, after freeze-drying, constitutes another protein fraction. Together, the two fractions constitute about half of the proteins present in the dehulled, defatted *Crambe* meal.

To develop applications with a higher added value, knowledge of the functional properties of the proteins, apart from their nutritional quality, is of prime importance. Therefore, the aim of this study was to evaluate some of the intrinsic biochemical and functional properties of these fractions.

EXPERIMENTAL PROCEDURES

Materials. Seed of *C. abyssinica* Hochst Ex. R.E. Fries was provided by Cebeco-Handelsraad B.V. (Rotterdam, The Netherlands). The Bio-Rad *DC* protein assay kit and the calibration kits for gel permeation chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) were supplied by Pharmacia (Uppsala, Sweden). Chicken egg white (whippable) was supplied by NIVE B.V. (Nunspeet, The Netherlands).

Preparation of meal. Dehulling, milling, and defatting (and heat treatment where applicable) were performed as outlined in the preceding paper (7).

Preparation of the protein fractions. The process described in the preceding paper (7) was followed. Briefly, the proteins from the dehulled, defatted meal were twice extracted at pH 11 (ratio meal to solvent 1:10 wt/vol). After centrifugation and filtration, the two extracts were combined. The extract was acidified to pH 5.5 where most protein precipitated. The precipitate was freeze-dried and then defatted to remove the remaining fat. This fraction had a protein content of 75% and is denoted as Fraction 1. The remaining supernatant was subjected to ultrafiltration, and the retentate was freeze-dried. This fraction had a protein content of 87% and is denoted as Fraction 2.

Gel permeation chromatography. The protein fractions were analyzed by using the Fast Protein Liquid Chromatography system (Pharmacia) and a Superose 6 HR 10/30 column (Pharmacia) with a separation range from 5000 to 5×10^6 Da. The column was equilibrated with 0.1 M sodium carbonate/sodium bicarbonate buffer of pH 9.2. The standards

*To whom correspondence should be addressed at present address: Lomvardou 81, 11474 Athens, Greece.

used for calibration of the column were thyroglobulin, ferritin, aldolase, ovalbumin, and ribonuclease of molecular weights 669,000, 440,000, 158,000, 43,000, and 13,700 Da, respectively. The absorbance was measured at 280 nm, and the flow rate was 0.3 mL/min. The lyophilized protein fractions were dispersed for analysis at 33 mg of protein in 5 mL water, adjusted to pH 11, vortexed to dissolve the material, and then made up to 10 mL by addition of buffer so that the final solution had a pH of 9.2. This procedure was necessary, especially for Fraction 1, to ensure maximal solubility. Then, the solutions were centrifuged for 15 min at 16,000 × *g*, and the supernatants were filtered through a 0.22-μm filter and applied on the column. The sample volume was 200 μL.

SDS–PAGE. The gel used was a 12.5% SDS homogeneous polyacrylamide gel (Pharmacia) with a separation range of 15,000 to 250,000 Da. The standards used for calibration of the gel were phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin with molecular weights of 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400 Da, respectively. The sample buffer used was 0.063 M Tris-HCL (pH 6.8) that contained 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 20% (vol/vol) glycerol, and 0.01% (g/vol) bromophenol blue. The concentration of the samples was 25 μg protein/mL buffer solution. Both standards and sample solutions were heated at 100°C for 10 min, cooled to room temperature, and centrifuged at 16,000 × *g* for 15 min; 1 μL of the supernatant was applied on the gel. Electrophoresis, staining, and destaining were performed on a Fast System Electrophoresis unit (Pharmacia) according to the instructions of the supplier.

Solubility. The protein fractions were dissolved in water at pH 11 to give a protein concentration of 1.5 mg/mL. NaCl was added, where applicable, in such an amount that the resulting solutions had molarities of 0.1, 0.5, and 1. Samples of varying pH were prepared from the pH 11 solutions by acidification. After standing for 1 h at room temperature, they were centrifuged at 16,000 × *g* for 15 min. The protein content of the supernatants was determined by the Bio-Rad *DC* protein assay. Solubility at pH 11 was assumed to be 100%, and the results were expressed relative to this value.

Foaming properties. Foaming properties were studied at several pH values with and without addition of NaCl according to the method described by Patel *et al*. (8). The parameters determined were FE (foam expansion), FVS (foam volume stability), and FLS (foam liquid stability).

Emulsifying properties. Emulsifying properties were determined by homogenizing 40 g of a 0.1% protein solution with 15 g soybean oil by means of an Ultra-Turrax (Janke and Kunkel GmbH, Staufen, Germany)at 13,000 rpm for 1 min. The turbidity of the resulting emulsion directly after homogenization was taken as an index for emulsifying activity. The ratio times 100 of the turbidity of the emulsion after standing 30 min at room temperature over the turbidity directly after homogenization was taken as an index for emulsifying stability. Turbidities were determined spectrophotometrically at 500 nm.

RESULTS AND DISCUSSION

Gel permeation chromatography. Figure 1 shows the elution profiles obtained for Fraction 1 and Fraction 2 on a Superose 6 HR column. For Fraction 1, three major classes of material can be distinguished, which are indicated by I, II, and III (Fig. 1B). Class I represents proteins with molecular weights much higher than that of thyroglobulin (669,000 Da). Class II represents proteins of varying molecular weights, ranging approximately from 669,000 to 13,700 Da. The third distinct peak, Class III, comprises proteins of molecular weights below 13,700 Da. The chromatogram of Fraction 2 differs clearly from that obtained from Fraction 1. Three distinct peaks can be seen, denoted by A, B, and C (Fig. 1C). Peak A comprises proteins with molecular weights ranging approximately from 440,000 to 158,000 Da. The proteins of peak B have molecular weights of about 13,700 Da, and proteins of peak C have molecular weights much less than 13,700 Da. Therefore, the isoelectric precipitation step of the isolation procedure (7) results in precipitation of all high-molecular-weight proteins. This is in agreement with the results of Lönnerdal *et al*. (9) who studied rapeseed protein isolates by molecular sieve chromatography. The high-molecular-weight proteins of Fraction 1 appeared only slightly on the chromatogram when Fraction 1 was dispersed directly in the buffer (results not shown). The same phenomenon was reported by

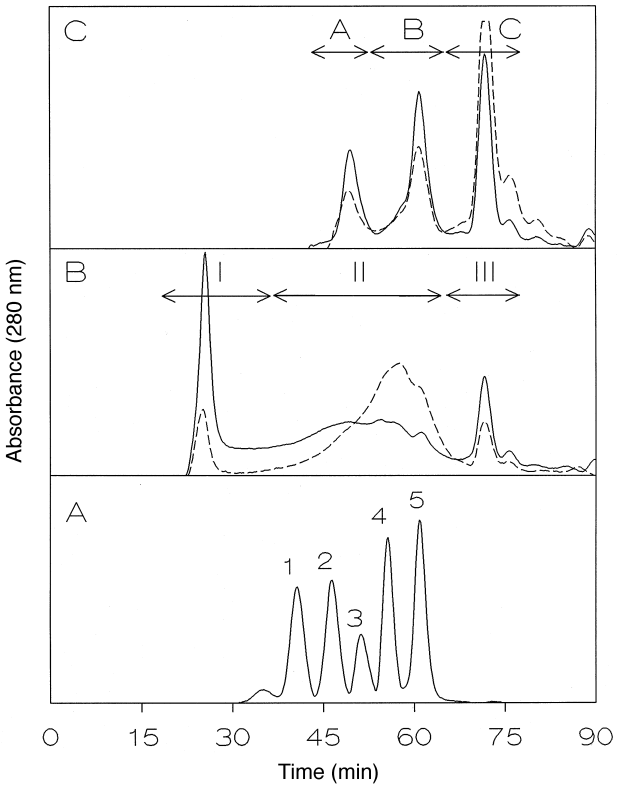

FIG. 1. Gel filtration chromatograms; (A) standards: 1 thyroglobulin (669,000 Da), 2 ferritin (440,000 Da), 3 aldolase (158,000 Da), 4 ovalbumin (43,000 Da), 5 ribonuclease (13,700 Da); (B) Fraction 1; (C) Fraction 2. Solid line: nonheated material; dotted line: heat-treated material.

Lönnerdal *et al*. (9) for rapeseed proteins; they attributed this to the fact that high-molecular-weight proteins did not easily redissolve. Dissolution of Fraction 1 at pH 11, followed by addition of buffer, increased the amount of the high-molecular-weight proteins present in the chromatogram. This could indicate that the presence of certain ions inhibits dissolution of these proteins, possibly through formation of aggregates. However, if the proteins are already dissolved, the presence of ions does not seem to have any significant effect.

Heat treatment (93°C for 30 min at a moisture content of 14%) had a pronounced effect on the proteins of Fraction 1. Not only is the recovery of Fraction 1 decreased (7) but also the relative amounts of proteins present in the three classes (Fig. 1). In particular, the relative amounts of high-molecular-weight proteins decreased and those of class II increased. This may be due to a decrease in the extractability of the high-molecular-weight proteins. Heat also affected the proteins of Fraction 2 but to a smaller extent than those of Fraction 1. Relatively more low-molecular-weight proteins were present in Fraction 2 after heat treatment.

SDS–PAGE. SDS–PAGE patterns, after reduction of disulfide bonds by mercaptoethanol, are shown in Figure 2. In general, both fractions consist of low-molecular-weight proteins, ranging mainly from 40,000 to less than 14,400 Da. Fraction 1 is much richer in proteins of about 20,000 Da, whereas Fraction 2 is richer in proteins in the range from somewhat above 20,000 to around 40,000 Da. SDS–PAGE analysis clearly indicates that the large proteins found by gel permeation chromatography are built up from smaller subunits. Furthermore, the same proteins are present in the starting meal, in the meal residue, and in the alkaline extract. This may indicate that protein extractability from *Crambe* meal is determined by their aggregation state. When the aggregates are too large, they are not extracted from the meal by water at alkaline pH.

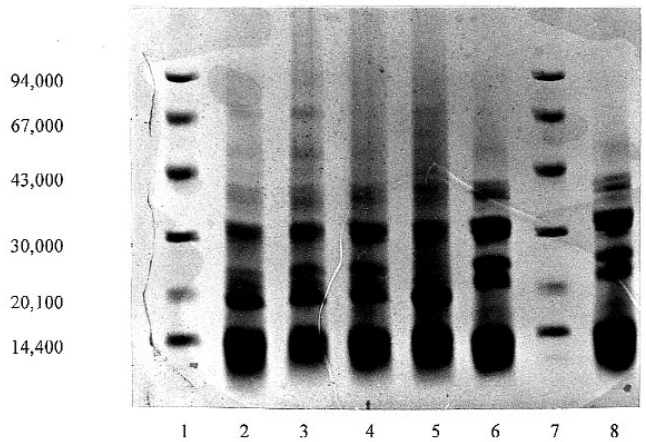

FIG. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12.5% polyacrylamide gel; lanes 1, 7: phosphorylase b (94,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da); 2: starting meal; 3: meal residue after extraction at pH 11; 4: protein extract at pH 11; 5: Fraction 1; 6, 8: Fraction 2.

Solubility. Solubility behavior provides a good indication of the potential applications of proteins. The solubility profiles of Fractions 1 and 2 as a function of pH are shown in Figure 3. Proteins present in Fraction 1 show minimal solubility at pH 4.5–6.5, where approximately 80% of the proteins precipitate. Fraction 1 was isolated by isoelectric precipitation at pH 5.5. The shape of the curve is similar to that of proteins from other oilseeds, such as rapeseed, soybean, and sunflower (10–12). On the other hand, the solubility of proteins from Fraction 2 was high (>80%) over the whole pH range tested. This behavior is similar to whey protein concentrates (13) and plasma proteins (14). For highly hydrophilic proteins, the increase in attractive forces at the isoelectric range is not sufficient to cause precipitation (15,16). Overall, the solubility profiles of both fractions resemble the solubility characteristics of rapeseed protein isolates, which were prepared in a similar way (precipitation–ultrafiltration) by Xu and Diosady (17).

Salts may alter the solubility of proteins by affecting protein–protein and protein–water interactions. Depending on concentration and the type of salt, solubility may increase or decrease. The effect of addition of sodium chloride on the solubility of proteins of Fraction 1 as a function of pH is shown in Figure 4. Generally, increasing the amount of NaCl increases protein solubility around the isoelectric range. At pH values >8, solubilities were similar except in 0.1 M NaCl, which was lower. At acidic pH values, addition of NaCl in amounts above 0.1 M decreases solubility markedly. One possible explanation of the effect of NaCl on protein solubility could be that, at the isoelectric point, NaCl exerts a salting-in effect (18). Also at higher alkaline pH values and higher ionic

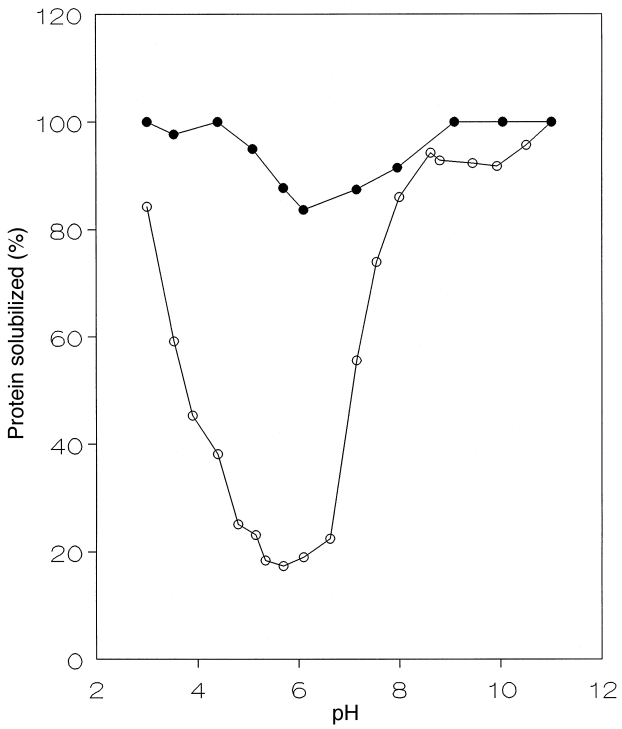

FIG. 3. Solubility of both fractions as a function of pH: (○) Fraction 1; (●) Fraction 2.

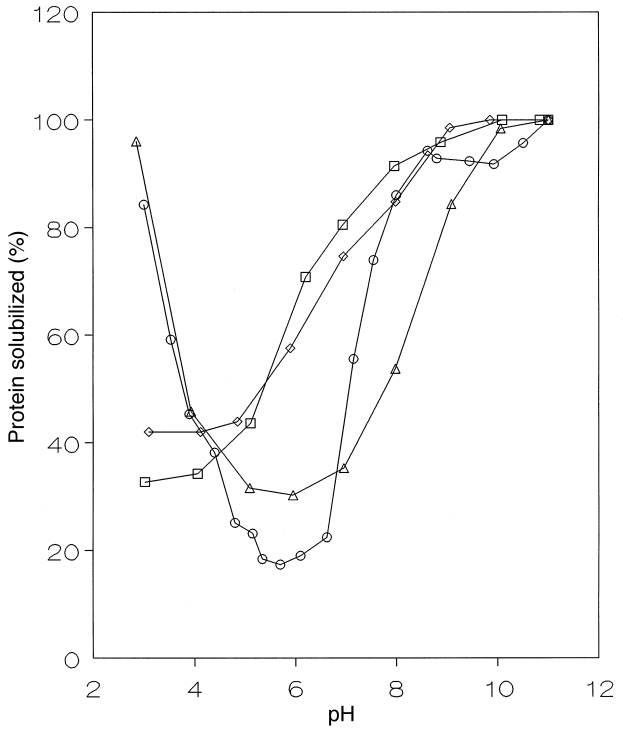

FIG. 4. Solubility of Fraction 1 as a function of pH and ionic strength: (○) salt-free; (△) 0.1 M NaCl; (◇) 0.5 M NaCl; (□) 1 M NaCl.

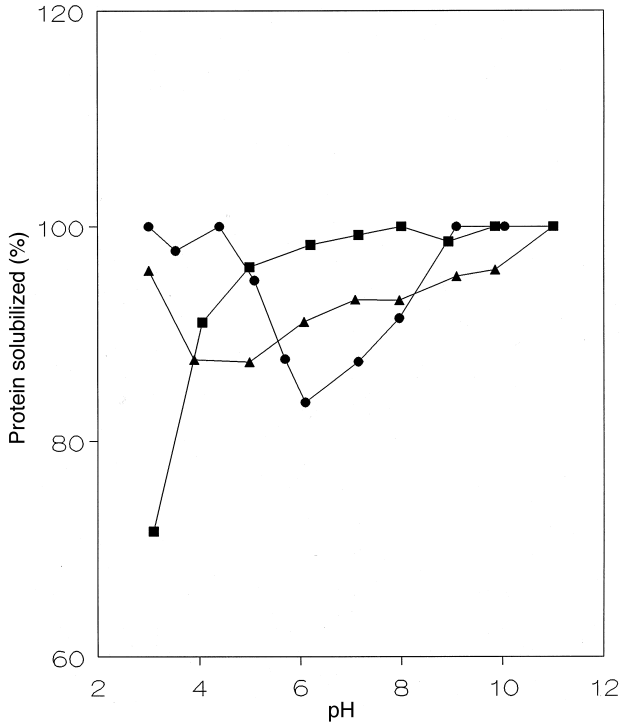

FIG. 5. Solubility of Fraction 2 as a function of pH and ionic strength: (●) salt-free; (▲) 0.1 M NaCl; (■) 1 M NaCl.

strength, NaCl seems to have a salting-in effect. At acidic pH values and increased ionic strength, the negatively charged chloride ions interact with the positively charged amino groups. This electrostatic shielding of the protein by the ions progressively decreases the net electrostatic repulsive interaction between the protein molecules and enhances hydrophobic protein–protein interaction, resulting in insolubilization (18). Similar results on the effect of NaCl on soy proteins were found by Hermansson (19) and Shen (20).

The effect of addition of NaCl on the solubility of proteins of Fraction 2 as a function of pH is shown in Figure 5. Addition of NaCl had only a minor effect. The solubilities were high (>80%) at all pH values and NaCl concentrations tested; the only exception was at pH 3 when a high amount of NaCl (1 M) was added.

Foaming properties. The foaming properties determined in this study are foam expansion (FE), which provides a measure of the ability of proteins to foam, and foam stability. The latter includes two aspects, namely, maintenance of the foam volume or foam volume stability (FVS) and the absence of draining of liquid from the foam or foam liquid stability (FLS). The foaming properties of Fractions 1 and 2 as a function of pH and ionic strength are shown in Table 1. For Fraction 1, when no NaCl was added, the foaming properties increased with increasing pH, especially at pH 9. Upon addition of 0.1 M NaCl, the same trend was also observed for FE and FLS, while values for FVS did not change markedly with increasing pH. At the highest ionic strength tested (0.5 M), there was nearly no effect of pH on the foaming properties, except that FLS was lower at pH 3. All foaming properties of Fraction 1 increased with increasing amount of salt. Foaming properties of Fraction 2 were only slightly affected by increasing pH or by addition of NaCl. Foaming properties of Fraction 2 were often superior to those of Fraction 1. Chicken egg white, which is commercially used for its foaming properties, showed the highest values at pH 5, which is close to the isoelectric point (21). Compared to chicken egg white, Fraction 1 performed better only at pH 9, while Fraction 2 was superior at almost every pH studied.

Many factors influence the foaming properties of proteins. These include protein type, method of preparation, composition, solubility, concentration, pH, the presence of salts, hydrophobicity, and of course, method of measurement. It is generally assumed that, for foam formation, proteins should be soluble in the aqueous phase and be capable of rapid unfolding at the air–water interface to form a cohesive layer around air bubbles. For foam stability, it is necessary that the protein film possesses intermolecular cohesiveness and a certain degree of elasticity to withstand local deformation (15,18,21). The cohesiveness, and hence the rigidity of protein films, is usually maximal at the isoelectric point where electrostatic repulsions between molecules are minimized (22). Several researchers observed maximal foaming ability (16,23), stability (24–26), or both (14,27,28) close to or at the isoelectric point. Others found maximal foam ability (29–31), stability (23,32), or both (11,14,33–35) away from the isoelectric range. Therefore, the relationship between pH and/or solubility and the foaming properties of proteins is still unclear. In our study, proteins of Fraction 2 had good foaming properties at all pH values, which is in accordance with their

TABLE 1
Foaming Properties of Fractions 1 and 2 as a Function of pH and Ionic Strength[a]

		Fraction 1			Fraction 2			Chicken egg white		
pH	M[b]	% FE	% FVS	% FLS	% FE	% FVS	% FLS	% FE	% FVS	% FLS
3	0	204 ± 8.5	72.3 ± 0.31	12.0 ± 0.56	620 ± 6.5	90.9 ± 0.48	30.5 ± 0.44	368 ± 10.5	71.2 ± 0.54	20.0 ± 0.49
3	0.1	440 ± 6.2	85.9 ± 0.45	20.0 ± 0.34	—	—	—	—	—	—
3	0.5	536 ± 7.0	90.3 ± 0.37	24.2 ± 0.41	660 ± 11.2	92.5 ± 0.42	34.8 ± 0.39	—	—	—
5	0	244 ± 6.5	77.9 ± 0.33	20.0 ± 0.48	660 ± 8.7	91.4 ± 0.34	26.3 ± 0.49	525 ± 7.9	91.3 ± 0.85	49.2 ± 0.53
5	0.1	388 ± 6.1	87.0 ± 0.52	32.6 ± 0.58	—	—	—	—	—	—
5	0.5	512 ± 8.4	91.0 ± 0.86	41.1 ± 0.76	632 ± 8.0	91.6 ± 0.55	26.3 ± 0.72	—	—	—
7	0	268 ± 6.3	78.3 ± 0.74	12.0 ± 0.42	660 ± 6.6	90.8 ± 0.83	30.5 ± 0.58	476 ± 8.3	85.1 ± 0.43	26.3 ± 0.73
7	0.1	464 ± 7.1	88.7 ± 0.68	30.5 ± 0.39	—	—	—	—	—	—
7	0.5	544 ± 8.9	92.7 ± 0.43	41.1 ± 0.65	620 ± 6.9	90.6 ± 0.58	24.2 ± 0.57	—	—	—
9	0	504 ± 7.5	90.9 ± 0.47	36.9 ± 0.82	696 ± 9.7	91.8 ± 0.39	34.7 ± 0.64	420 ± 6.8	81.4 ± 0.64	20.0 ± 0.48
9	0.1	580 ± 9.3	92.5 ± 0.61	41.1 ± 0.73	—	—	—	—	—	—
9	0.5	580 ± 10.1	90.8 ± 0.38	45.3 ± 0.47	660 ± 7.6	90.6 ± 0.77	32.6 ± 0.46	—	—	—

[a]Mean ± standard deviation, $n = 3$.
[b]Molarity of added salt solution. FE, foam expansion; FVS, foam volume stability; FLS, foam liquid stability.

solubility profile, implying that solubility could be an important factor. On the other hand, proteins of Fraction 1, which were soluble at both alkaline and acid pH values, showed the best foaming properties only at alkaline pH values, indicating that, for some proteins, charge also plays an important role in foaming behavior.

Addition of salts may alter foaming properties of proteins by affecting solubility, viscosity, unfolding, and aggregation of proteins (21). Their effect varies with ion species and concentration (16,30). Some studies have shown that, upon addition of NaCl, foaming ability increases (16,27,29,31,33), whereas foam stability decreases (34) or increases (27,29). However, in these investigations, addition of NaCl was not studied as a function of pH. In our studies, the foaming properties of Fraction 2, like solubility, were not affected markedly by addition of NaCl. The foaming properties of proteins from Fraction 1 were also not affected by addition of a small amount of NaCl, although the same amount of NaCl increased their solubility in the isoelectric range. On the other hand, larger amounts of NaCl caused a significant improvement in foaming properties. In addition, the fact that high amounts of NaCl decreased solubility at acidic pH values points to variables other than solubility being also important for the foaming behavior of these proteins.

Emulsifying properties. The emulsifying properties determined in this study are the emulsifying activity and emulsifying stability. The former is related to the ability of a protein to form an emulsion, i.e., to assist in the dispersion of oil into small globules that are homogeneously distributed throughout the continuous aqueous phase. The emulsifying stability is related to the ability of a protein to maintain homogeneity of the oil-in-water emulsion on storage. Emulsifying activity and stability of Fractions 1 and 2 as a function of pH are shown in Figures 6 and 7. Both properties were markedly affected by pH. For both fractions, emulsifying activity paralleled emulsifying stability and sharply decreased near the isoelectric point. Both fractions were more efficient in emulsifying the oil at alkaline pH values.

Many factors influence emulsions, among others the equipment used, oil phase volume, pH, solubility, ionic strength, protein concentration, and surface properties of the protein. A positive correlation is often found between protein solubility and emulsion formation because diffusion toward and adsorbance at the oil–water interface are necessary (21). Emulsifying ability of soluble proteins depends upon the surface hydrophobicity of the protein as well as on the hydrophilic-lipophilic balance, which is affected by pH (31,36). At the isoelectric point, the protein may aggregate and destabilize the interfacial membrane; on the other hand, protein adsorption and viscoelasticity at the oil–water interface, being

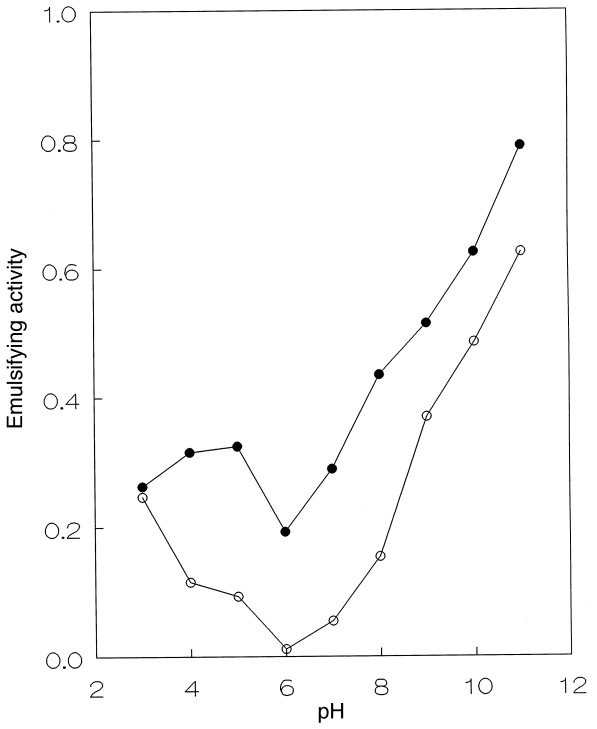

FIG. 6. Emulsifying activity of both fractions as a function of pH: (○) Fraction 1; (●) Fraction 2. Absorbance = 500 nm.

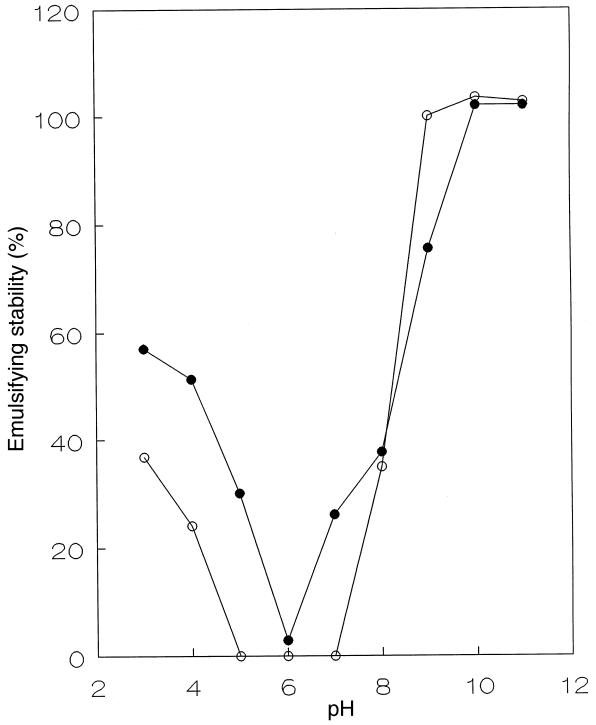

FIG. 7. Emulsifying stability of both fractions as a function of pH: (○) Fraction 1; (●) Fraction 2.

maximal at this pH, act in the opposite direction (31). Therefore, the net charge at the interface may impede or facilitate emulsifying activity of proteins, and this fact partly accounts for the conflicting experimental data, i.e., some proteins have optimal emulsifying properties at the isoelectric point while others perform better at other pH values. The effect of pH found in our study is similar to that reported by other investigators for other proteins (31,37–40). However, it is different from that reported by Wang and Kinsella (41), who found for alfalfa leaf proteins maximal emulsification in the vicinity of the isoelectric range. Although Fraction 1 has high solubility at acidic pH values, it did not emulsify well in this range. Fraction 2, which is soluble at all pH values tested, performed well only at alkaline values. Therefore, for *Crambe* proteins, pH seems to play a more important role than solubility.

This research has shown that the two protein fractions derived from *Crambe* oilseed comprise proteins with distinctive molecular weight distributions. These differences may partly account for the differences in functionality. Furthermore, the two fractions obtained show interesting functional properties, especially foaming properties. These properties may be exploitable in potential food applications (e.g., as a substitute for chicken egg white), and/or in nonfood applications, such as foam concrete, foamed packaging material, and foamed disposables.

ACKNOWLEDGMENT

Financial support by the Commission of the European Communities (DG XII) is gratefully acknowledged.

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[Received April 7, 1997; accepted September 9, 1997]