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Fluorescence due to interactions of oxidizing soybean oil and soy proteins

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Abstract

Soy proteins with soybean oil (9:1,w/w) were stored at 60°C to investigate the changes in intrinsic fluorescence during oxidation. The front-surface fluorescence of the oxidized samples showed excitation and emission maxima at 355 and 440 nm respectively. The fluorescent compounds were soluble in the organic layer of the chloroform–methanol (2:1,v/v). The solution fluorescence showed an excitation maximum at 365 nm and an emission maximum at 450 nm, and the intensity increased during storage. The interactions of oxidizing soybean oil and soy proteins also resulted in decreases of protein solubility, soluble protein hydrophobicity, and free amino groups of proteins. With an antioxidant (BHT) addition, the changes in fluorescence and in protein properties were inhibited. The intensity of the solution fluorescence showed high correlation with TBA value ($r = 0.968$) and protein solubility ($r = -0.979$), which could serve as an indicator for oxidative deterioration of soy proteins and soybean oil systems. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Soybeans are important sources of proteins and vegetable oil. Soybeans and soy proteins are extensively used in Oriental foods (Chen, 1992; Golbitz, 1995; Lusas & Riaz, 1995). The oxidation of lipids, however, leads to the development of off-flavour and generates certain oxidation products (Nawar, 1985). The oxidation products can further react with some food constituents, especially proteins, and cause functional property changes (Cheftel, 1977; Hidalgo & Kinsella, 1989).

In previous investigations, the lipids associated with soy protein isolates (SPI) were found to contribute to protein insolubility and protein oxidation (Boatright & Hettiarachchy, 1995a). Adding antioxidants to SPI during processing resulted in a marked increase in protein solubility (Boatright & Hettiarachchy, 1995b). The solubility of proteins correlates with other functional properties, such as gelation, viscosity, emulsifying activity and foaming (Kinsella, 1979). Consequently, the effects of lipid oxidation on soy protein functionalities are important. Appropriate and reliable methods for assessment of lipid oxidation and protein solubility in soy protein products are of interest.

The peroxide value and 2-thiobarbituric acid (TBA) value are commonly used as indices of lipid peroxidation in foods (Gray, 1978; Melton, 1983; Frankel, 1993). However, they are not necessarily correlated quantitatively with lipid peroxidation in some foods because hydroperoxides and TBA-reactants are labile and easily react with some food constituents (Chipault & Hawkins, 1971; Williams, Field, Miller, & Welke, 1983; Hasegawa, Endo, & Fujimoto, 1992, 1993). In addition to peroxide and TBA values, fluorescent products of reaction between oxidizing lipids and proteins have been used for assessment of lipid oxidation in foods (Kamarei & Karel, 1984; Pikul, Leszczynski, Bechtel, & Kummerow, 1984; Nolan, Bowers, & Kropf, 1989; Pikul & Kummerow, 1990). Fluorescent chromophores are either lipid- or water-soluble,or insoluble, and a major portion of the fluorescent compounds in oxidizing biological tissues are extractable into a chloroform–methanol mixture (Fletcher, Dillard, & Tappel, 1973; Nakhost & Karel, 1989). Hence, the fluorescence intensity in the lipid-soluble phase of the chloroform–methanol has been used for evaluating lipid oxidation in foods (Dillard & Tappel, 1973; Kamarei & Karel, 1984; Bouzas, Kamarei, & Karel, 1985; Liang, 1996). Fluorescence spectroscopy offers several inherent advantages for the characterization of molecular reaction and interactions, and is 100–1000 times more sensitive than spectrophotometric techniques (Dillard & Tappel, 1971; Strasburg & Ludescher, 1995).

Model systems of soy proteins and soybean oil were designed to investigate the change of intrinsic fluorescence during oxidation. A fluorescence method was used

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to assess the extent of lipid oxidation as well as protein solubility.

2. Materials and methods

2.1. Materials and sample preparation

Soy protein isolate (Supro 620) was obtained from Protein Technologies International (St. Louis, MO, USA). Soybean oil was bought from a local market. Butylated hydroxytoluene (BHT), Folin-Ciocalteu's phenol reagent, ammonium thiocyanate and 2-thiobarbituric acid were purchased from E. Merck (West Germany). Quinine sulfate dihydrate and ferrous chloride (anhydrous) were supplied by Fluka (Buchs, Switzerland). Sodium dodecyl sulfate(SDS), fluorescamine and the hemimagnesium salt of 8-anilino-1-naphthalene-sulfonic acid were purchased from Sigma Chemical Co.(St. Louis, MO, USA). Other chemicals used were of analytical grade and were purchased from reliable commercial sources.

Model system PO was prepared by mixing soy protein isolate and soybean oil (9:1,w/w) in hexane to form a paste, and then dried at 40°C under reduced pressure. Model system POBHT was prepared in the same manner except 200 ppm BHT was added on the basis of soybean oil.

2.2. Storage and treatment of samples

Samples of PO (15 g) and POBHT (15 g) were stored under air in the dark at 60°C. For comparison purposes, soy protein isolate (SPI) was stored as control. At various time intervals, 0.5 g of each of the samples was withdrawn and vortexed with 10 ml chloroform–methanol (2:1,v/v), incubated at room temperature for 10 min, and filtered through Toyo No. 1 filter paper (Toyo Roshi, Kaisha, Ltd. Japan). The clear filtrate (the CM extract) was adjusted to 10 ml with additional chloroform–methanol. Aliquots of the CM extracts were subjected to peroxide value, TBA value and fluorescence assays. The defatted samples were employed for the determination of the protein solubility, free amino group content and soluble protein hydrophobicity. Experiments were performed twice and all data were duplicate measurements.

2.3. Front-surface fluorescence measurement

The front-surface fluorescence of PO was measured by dispersing in fluorescence-free glycerin/water (1/1, v/v) (Munck, 1993). The spectra were obtained by a F-2000 spectrofluorometer (Hitachi Ltd, Tokyo, Japan) equipped with a front-surface sample cell holder. The excitation light, provided by a xenon lamp, was incident at an angle of 37 degrees to lower reflected light. The excitation spectra of the samples were scanned from 220 to 400 nm with the emission wavelength fixed at 440 nm. The emission spectra were scanned from 400 to 600 nm with the excitation wavelength fixed at 355 nm. The spectra were measured at conditions as follows: scan speed, 240 nm/min; response, 0.5 s; bandpass, 20 nm; photomultiplier voltage, 400 V.

2.4. Transmission fluorescence of the CM extracts

Six millilitres of the CM extract were transferred to a centrifuge tube, mixed well with 2 ml distilled water, and subsequently centrifuged at 1000×**g** for 10 min. The solvent layer was collected and used to obtain the fluorescence spectra and fluorescence intensity.

The spectra of the solution fluorescences were determined by a Hitachi F-2000 spectrofluorometer with a quartz cuvette (10 mm pathlength) in the conventional right-angle orientation. The excitation spectra of the solvent layers were scanned from 220 to 400 nm with the emission wavelength fixed at 450 nm. The emission spectra were scanned from 400 to 600 nm with the excitation wavelength fixed at 365 nm. The fluorescence intensity was determined at an excitation wavelength of 365 nm and emission wavelength 450 nm. The intensity was expressed as a relative ratio for a standard solution of 1 ppm quinine sulfate in 0.1 N H_2SO_4 (Gillespie, 1985).

2.5. Peroxide value

Peroxide values were determined using the ferric thiocyanate method (Egan, Krik, & Sawyer, 1981) on the CM extracts. Each 0.1 ml of the CM extract was mixed with 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of ferrous chloride solution (20 mM in 0.35% HCl), and then diluted with 4.7 ml chloroform-methanol. The absorbance of the mixture was determined at 500 nm. The CM extract of SPI was used as blank. Results were expressed as milliequivalents peroxide per kilogram of sample.

2.6. TBA value

Thiobarbituric acid values were determined on the CM extracts according to the method of Nair and Turner (1984). The TBA value was expressed as nanomoles of malondialdehyde per gram of sample by using the extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ for malondialdehyde.

2.7. Free amino group content

Five milligrams of the defatted sample was weighed and stirred into 10 ml of 4% SDS in 0.1 M phosphate buffer (pH 8.0). After incubating in a 100°C water bath for 10

minutes, the dispersion was cooled to room temperature. An aliquot (50 µl) of the dispersion was employed for the determination of free amino group content according to the method of Bohlen, Stein, Dairman, and Udenfriend (1973).

2.8. Protein solubility

Fifty milligrams of the defatted sample was treated with 5 ml of 0.1 M NaCl in a centrifuge tube. After stirring for 1 h by sonication, the dispersion was centrifuged, and filtered through Toyo # 1 filter paper. Soluble protein in the filtrate was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951).

2.9. Soluble protein hydrophobicity

The above soluble protein solutions were also used to determine the soluble protein hydrophobicity by the 8-anilino-1-naphthalenesulfonate (ANS) binding method. Measurements were performed according to the method of Kato and Nakai (1980) in the absence of SDS. Each 0.2 ml protein solution was diluted with 1.8 ml 0.01 M phosphate buffer (pH = 7), and 10 µl ANS (8 mM in 0.1 M phosphate buffer, pH 7) solution was then added. Fluorescence intensity (FI) was measured at ex. 390 nm and em. 470 nm, and expressed as a relative ratio for a standard solution of 10 µl ANS in 2 ml methanol. The ratio of FI to protein solubility was used as an index of soluble protein hydrophobicity.

3. Results and discussion

The front-surface fluorescence of the samples were assayed by reflectance spectrofluorometry. The spectra of the oxidized PO had an excitation maximum at 355 nm and an emission maximum at 440 nm (Fig. 1). For obtaining the excitation spectra of all samples, the emission wavelength was set at 440 nm; for obtaining the emission spectra, the excitation wavelength was set at 355 nm. In contrast, the front-surface fluorescence of the fresh PO showed two peaks in the excitation spectrum around 280 nm (with maximum intensity) and 345 nm. The fluorescence spectra of soy proteins and soybean oil mixtures were quite broad, and the fluorescence intensity at excitation 355 nm and emission 440 nm increased markedly during oxidation. The spectra could be the sum of several spectra of different intrinsic fluorescent compounds.

The front-surface fluorescence spectra of the samples after extraction with chloroform-methanol (2:1, v/v) are shown in Fig. 2. The fluorescence intensity of the defatted oxidized PO was reduced to about the same level as that of the defatted fresh PO. The results indicated that the fluorescence compounds formed are mostly soluble in chloroform–methanol. The excitation spectrum of the defatted oxidized PO showed two peaks around 280 and 350 nm, which was similar to that of the defatted fresh PO. The intensity at 350 nm was slightly higher than that of the defatted fresh PO, which indicated that some oxidation products were insoluble. On the other hand, the intensity at 280 nm was lower than that of the defatted fresh PO. The excitation peak around 280 nm was mainly due to the absorption of aromatic groups of proteins (Undenfriend, 1962). In theory, the shape of the excitation spectrum should be identical to the absorption spectrum of the sample and independent of the wavelength at which the emission monochromator is set. This is not always the case, however, principally due to instrumental artifacts and sample environment (Gillespie, 1985). As shown in Fig. 2, the oxidizing soybean oil appeared to react with soy proteins. The interacton products, containing the fluorescence compounds, are mostly soluble in chloroform-methanol reagent. Therefore, the intensity at 280 nm decreased in the defatted oxidized PO.

The organic layers from the CM extracts were also studied by transmission spectrofluorometry. Scanning of well-oxidized PO extract indicated that excitation at

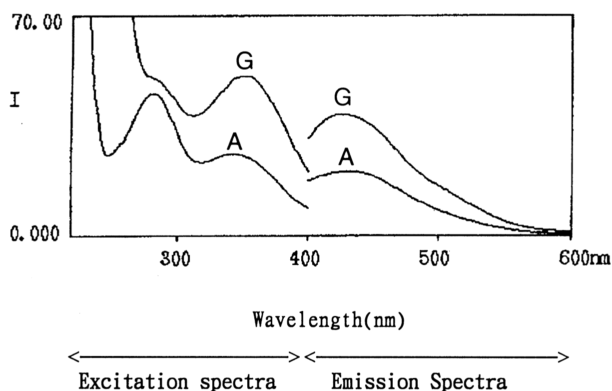

Fig. 1. Front-surface fluorescence spectra of the soy proteins–soybean oil mixtures. A: fresh; G: oxidized (60°C, 286 h).

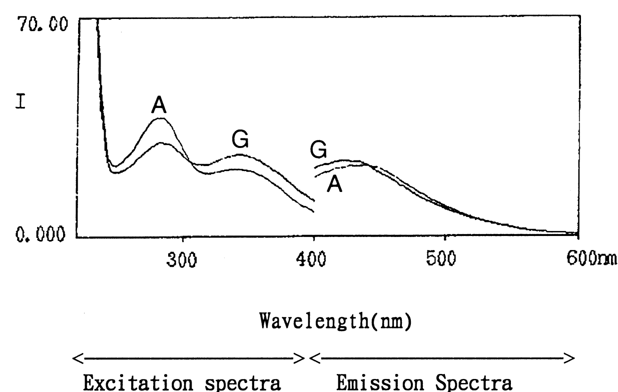

Fig. 2. Front-surface fluorescence spectra of the soy proteins–soybean oil mixtures after extraction with chloroform–methanol. A: fresh; G: oxidized (60°C, 286 h).

365 nm resulted in a maximum emission at 450 nm. When emission wavelength was set at 450 nm, the excitation spectrum showed a maximum intensity peak at 365 nm and a small peak at 275 nm. Consequently, for obtaining the excitation spectra of PO extract during oxidation, the emission wavelength was set at 450 nm and for obtaining the emission spectra, the excitation wavelength was set at 365 nm. Fig. 3 shows the variations of excitation and emission spectra of PO extracts during storage for up to 286 h. Unoxidized sample showed a single excitation peak with maximum wavelength around 310 nm. As the extent of oxidation proceeded, the intensity at 310 nm decreased while those at 365 and 275 nm increased. The growing excitation peak around 275 nm could further demonstrate that the oxidizing soybean oil reacted with soy proteins. The fluorescent compounds formed were soluble in the organic layer of the chloroform–methanol extraction. In the emission spectra, there was only one broad single peak whose intensity gradually increased upon oxidation while its maximum wavelength shifted from 440 toward 450 nm.

The solution fluorescence at ex. 365 nm and em. 450 nm was used to estimate the extent of lipid oxidation. The fluorescence intensity of different samples taken at successive stages of storage are shown in Fig. 4. Soy protein isolate did not build up any fluorescence products. The intensity of PO did not increase until the storage had proceeded for at least 90 h. However, the intensity increased significantly with storage time after the initial lag period. With the addition of an antioxidant, the fluorescence intensity of POBHT was effectively reduced.

The peroxide value and TBA value of the samples were measured on the chloroform-methanol extracts (Figs. 5 and 6). The peroxide value of PO increased for 142 h during storage and then decreased gradually. The peroxide value of POBHT reached a maximum much more slowly than did PO. During the hydroperoxide accumulation period, the fluorescence intensity increased slowly as shown in Fig. 4. Therefore, the fluorescence intensity of PO and POBHT were still negligible for up

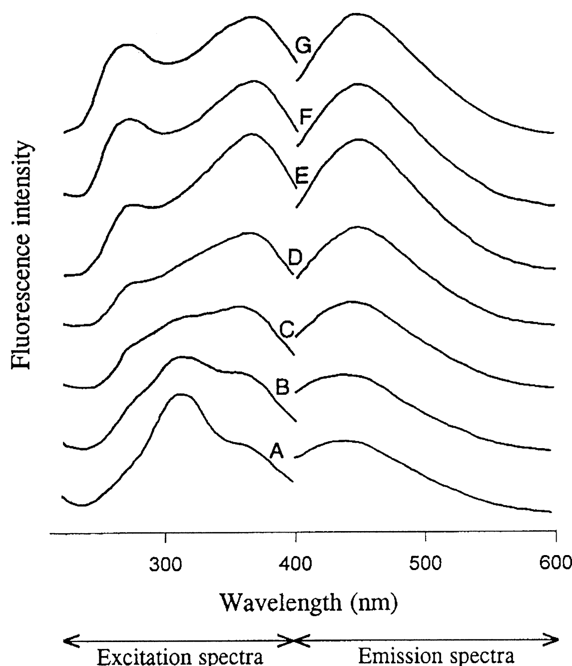

Fig. 3. Transmission fluorescence spectra of the organic layers from chloroform–methanol extractions of soy protein–soybean oil mixtures during oxidation at 60°C in the dark. A, 0 h; B, 44 h; C, 89.5 h; D, 119 h; E, 165 h; F, 213 h; G, 286 h.

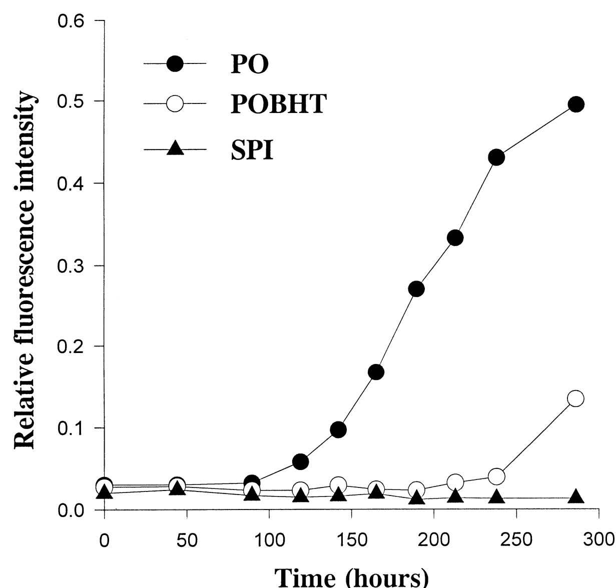

Fig. 4. Increase of fluorescence intensity (ex. 365 nm, em. 450 nm) in the organic layers of chloroform–methanol extractions during storage at 60°C in the dark.

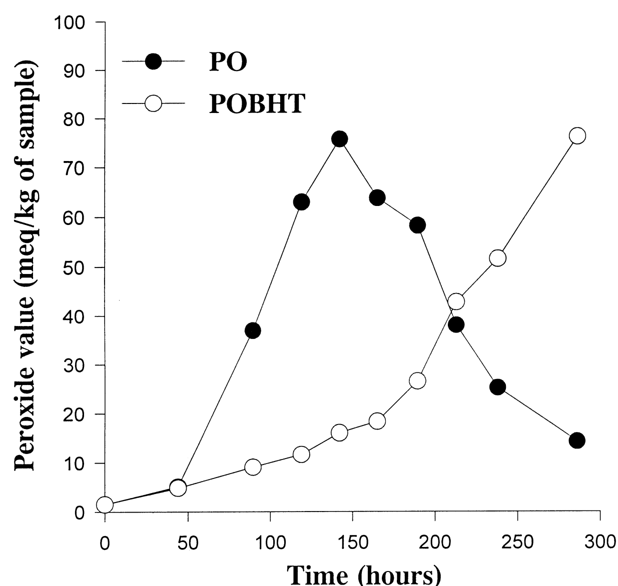

Fig. 5. Time courses of peroxide value of the samples during storage at 60°C in the dark.

to 100 and 250 h, respectively. After the initial lag periods, the fluorescence intensity increased in proportion to hydroperoxide decomposition products as indicated by TBA values (Fig. 6). These observations demonstrated that the fluorescent products might mainly derive from the interaction of soy proteins and thiobarbituric acid-reactive substances.

The free amino group content in samples decreased, throughout the storage period, with the extent of lipid oxidation (Fig. 7). The retention of free amino groups in POBHT was higher than PO. The loss of free amino groups could be inhibited by the addition of an antioxidant. The fluorescence spectra of oxidized PO (365 and 450 nm) were similiar to those of Schiff base structures (370 and 450 nm; Chio & Tappel, 1969), which demonstrated that TBA-reactive substances might react with free amino groups of proteins to form the Schiff base type of fluorescence products.

The interaction of oxidizing soybean oil with soy proteins also contributed to protein insolubility. The solubility of the defatted PO decreased to 79% of the original after 286 h of storage (Fig. 7). The POBHT, with the addition of an antioxidant, showed significant increase of protein solubility. Studies of the removal and sebsequent reintroduction of lipids, during soy protein isolate (SPI) processing, also demonstrated that lipids contribute to SPI insolubility (Boatright & Hettiarachchy, 1995c).

The effect of lipid oxidation on soluble protein hydrophobicity was examined. The hydrophobicity decreased throughout the storage period (Fig. 7). The addition of an antioxidant also inhibited the change. Hence, lipid oxidation resulted in both decreases in solubility and hydrophobicity. A decrease in protein surface hydrophobicity resulting from protein oxidation was observed during soy protein isolate processing (Boatright & Hettiarachchy, 1995b). Results in this study suggested that lipid oxidation products might react with the hydrophobicity groups of proteins to produce lipid-soluble fluorescence products, which resulted in decreases in soluble protein hydrophobicity of the defatted samples. Hence, the formation of fluorescence products and the loss of protein hydrophobicity could be inhibited with the use of antioxidants.

The resulting intrinsic fluorescence products were examined to correlate with the extent of lipid oxidation and protein solubility. The fluorescence intensity showed a high correlation with TBA value ($r = 0.968$)

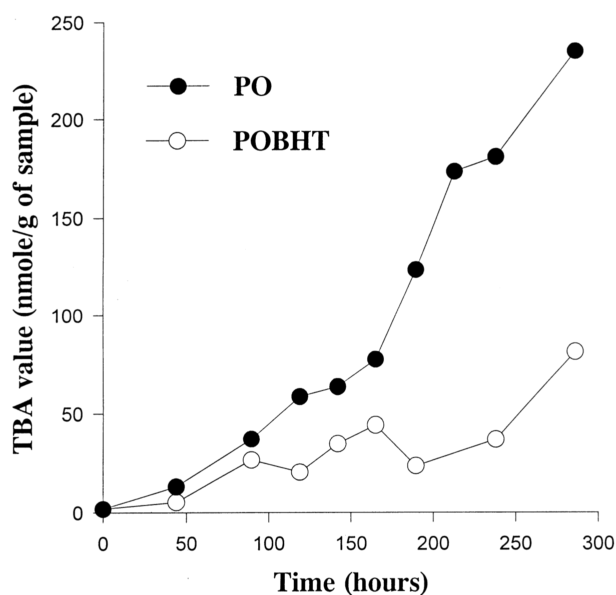

Fig. 6. Time courses of TBA value of the samples during storage at 60°C in the dark.

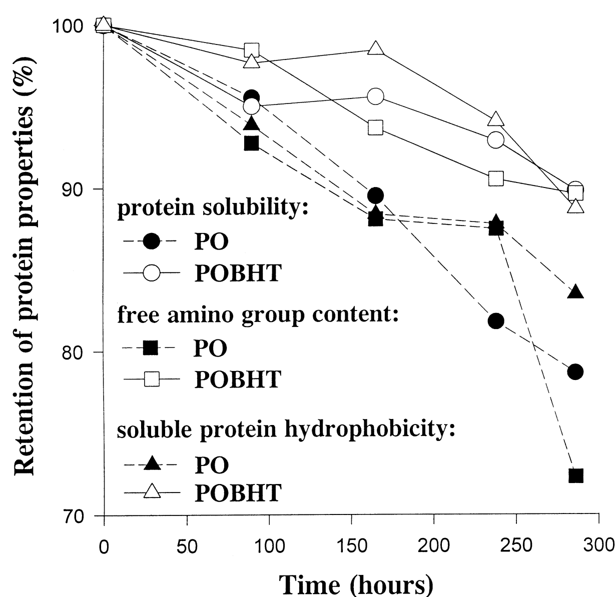

Fig. 7. Retention of protein properties in the samples during storage at 60°C in the dark.

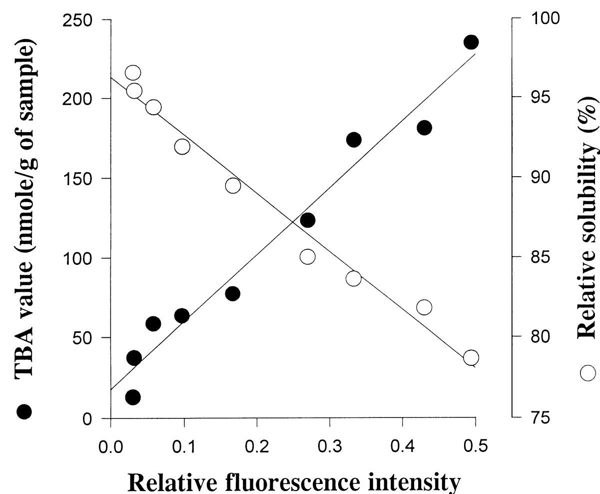

Fig. 8. Relationships of TBA value and protein solubility to the fluorescence intensity during storage of soy protein–soybean oil mixtures at 60°C in the dark.

and protein solubility ($r = -0.979$), respectively (Fig. 8). The solution fluorescence at ex. 365 nm and em. 450 nm was appropriate for evaluating lipid oxidation as well as protein solubility in soy proteins and soybean oil systems.

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