JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stability of Isoflavones in Soy Milk Stored at Elevated and Ambient Temperatures

BIANNA EISEN, YAEL UNGAR, AND EYAL SHIMONI*

Department of Food Engineering and Biotechnology, Technion—Israel Institute of Technology, Haifa 32000, Israel

Soy isoflavones are widely recognized for their potential health benefits. The increased use of traditional and new food products calls for the assessment of their stability during processing and storage. The present study examines the stability of genistein and daidzein derivatives in soy milk. Soy milk was stored at ambient and elevated temperatures, and the change in isoflavone concentration was monitored with time. Genistin loss in time showed typical first-order kinetics, with rate constants ranging from 0.437−3.871 to 61−109 days^{-1} in the temperature ranges of 15−37 and 70−90 °C, respectively. The temperature dependence of genistin loss followed the Arrhenius relation with activation energies of 7.2 kcal/mol at ambient temperatures and 17.6 kcal/mol at elevated temperatures. At early stages of soy milk storage at 80 and 90 °C, the 6″-*O*-acetyldaidzin concentration increased, followed by a slow decrease. The results obtained in this study can serve as a basis for estimating the shelf life of soy milk as related to its genistin content.

KEYWORDS: Isoflavones; stability; shelf life; soy milk

INTRODUCTION

Soy isoflavones belong to a subclass of flavonoids, and are often also referred to as phytoestrogens because of their ability to interact with estrogen receptors in cells. These phytoestrogenic isoflavones have gained attention in the past decade due to their potential protective or preventive activity against a number of diseases such as cardiovascular diseases, cancers, and osteoperosis (*1*−*5*).

The isoflavone content in soy-based foods depends on the specific product, its solids content, and processing and storage conditions. For example, the content of daidzein and three other isoflavones increased when soy sauce was produced from whole soybeans rather than defatted beans (*6*). Wang and Murphy (*7*) evaluated the concentration and distribution of isoflavones in 29 commercial soybean foods, categorized into soy ingredients, traditional soy foods, and second-generation soy foods. They identified 12 isomers, 3 aglycons (daidzein, genistein, glycitein) and 9 glucosides (daidzin, genistin, glycitin, 6″-*O*-acetyldaidzin, -genistin, and -glycitin, 6″-*O*-malonyldaidzin, -genistin, and -glycitin). High-protein soy ingredients contained similar concentrations compared with unprocessed soybeans (except alcohol-leached soy concentrate). Traditional nonfermented soybean foods had greater levels of glycosides, while in contrast, greater levels of aglycons were found in fermented foods. However, the second-generation soy foods contained only 6−20% of the isoflavones of whole soybeans. They concluded that the variety of soybean, method of processing, and addition of other components affect the retention and distribution of isoflavone isomers in soy foods. Murphy et al. (*8*) evaluated isoflavones in 63 retail and institutional soy foods, and reported levels from 1 μg/g in soy sauce to 540 μg/g in tempeh. Hence, the concentration and chemical structure of soy isoflavones in foods is highly dependent on the raw material and processing and storage conditions.

Very few studies have examined the effect of food processing on the isoflavone content, and on their chemical structure. Wang et al. (*9*) reported that heating daidzein and genistein conjugates under acid conditions (1−3 M HCl) released free isoflavones. They also showed that genistein was further degraded, and that its degradation occurred earlier as the acid concentration increased. When standard genistein was mixed with dextrose, fructose, maltose, and sucrose, it formed conjugates with very high UV absorption at 254 nm. The amount of these conjugates was proportional to the amount of sugar added (*9*).

High temperature and pressure reduced the total isoflavone content in corn−soy mixtures (*10*). The effects of processing techniques on the distribution of isoflavones were also investigated in the manufacturing of tempeh, soymilk, tofu, and protein isolate (*11*). They found that manufacturing steps causing significant losses of isoflavones were as follows: soaking (12%) and heat processing (49%) in tempeh production, coagulation (44%) in tofu processing, and alkaline extraction (53%) in soy protein isolate production. Malonyldaidzin and malonylgenistin decreased after soaking and cooking in the production of tempeh, soymilk, and tofu. Concomitantly, 6″-*O*-acetyldaidzin and 6″-*O*-acetylgenistin were generated during thermal processing. Alkaline extraction in protein isolate processing caused the generation of daidzein and genistein, probably through alkaline hydrolysis. Wang and Murphy (*11*) concluded that processing

* To whom correspondence should be addressed. Phone: +972-4-8292484. Fax. +972-4-8320742. E-mail: eshimoni@tx.technion.ac.il.

10.1021/jf025783h CCC: $25.00
Published on Web 03/12/2003

significantly affects the retention and distribution of isoflavones in food. However, to date, there are no data available about the kinetics of these changes and the factors affecting them.

Coward et al. (*12*) studied the chemical modification of isoflavones in soy foods during cooking and processing. Analysis of soy food products revealed that defatted soy flour that had not been heat treated consisted mostly of 6″-*O*-malonylglycitin conjugates; in contrast, toasted soy flour contained large amounts of 6″-*O*-acetyl-*β*-glucoside conjugates, formed by heat-induced decarboxylation of the malonate group to acetate. Soy milk and tofu consisted almost entirely of *β*-glucoside conjugates; low-fat versions of these products were markedly depleted in isoflavones. Alcohol-washed soy protein concentrates contained few isoflavones. Isolated soy protein and textured vegetable protein consisted of a mixture of all three types of isoflavone conjugates. Baking or frying of textured vegetable protein at 190 °C and baking of soy flour in cookies did not alter the total isoflavone content, but there was a steady increase in *β*-glucoside conjugates at the expense of 6″-*O*-malonylglycitin conjugates.

Acetylglucoside isoflavones were identified in toasted soy flakes by Farmakalidis and Murphy (*13*). These authors suggested that the higher content of these conjugates in 80% aqueous acetonitrile extracts compared with 80% aqueous methanol extracts was a function of the greater solubilizing power of the first. Others reported 6″-*O*-malonylglucoside conjugates to be the predominant form of isoflavones in soybean hypocotyl and cotyledon (*14*). These authors, however, suggested that the glucosides reported in many other studies that used hot aqueous alcohol extraction are products of de-esterification caused by the heat. Barnes et al. (*15*) showed that the composition of isoflavone conjugates in soybeans and soy products is complex, and pointed out the potential implication of the chemical form on isoflavone metabolism, bioavailability, and biological activity. It was their conclusion that studies are urgently needed to address this issue. The objective of the reported research was to determine the stability of genistin, the major isoflavone in soy milk, during storage at ambient and elevated temperatures.

MATERIALS AND METHODS

Soy Milk Samples. Soy milk used in the present study was UHT soy milk in Tetra Brik aseptic packaging. The composition of this specific soy milk (as stated by the manufacturer) was 3.6% protein, 0.3% carbohydrates (0.1% sugars), 2.1% fat, and 1.2% fiber, with pH 6.6. All samples used were of the same batch as stated by the manufacturer. After purchasing, all samples were stored at 4 °C until use.

Storage Tests. Samples of soy milk (5 mL) were aseptically withdrawn from the original packaging into 14 mL disposable tubes (Miniplast, Ein-Shemer, Israel). Tubes were incubated at various temperatures, and triplicates were withdrawn at predetermined times for isoflavone extraction and analysis. The effect of temperature on the reaction rate was studied by determining the reaction rate at three temperatures in two temperature ranges. For the low-temperature range, samples were incubated at 15, 25, and 37 °C, and for the high-temperature range at 70, 80, and 90 °C. The incubation at low temperatures was up to 129 days, and at the elevated temperatures up to 35 (80 and 90 °C) and 59 (70 °C) days. For the low temperatures, samples were taken after 0, 5, 12, 40, 72, 106, and 129 days. Samples were withdrawn from the elevated temperatures at 0, 2, 5, 12, 14, 16, 19, and 21 days at 80 and 90 °C, and also at 35 and 42 days at 70 °C. The Arrhenius relation was used to model the temperature effect on the reaction rate constant.

Isoflavone Analysis. Isoflavones were extracted from soy milk samples by the addition of 8 mL of methanol to a 2 mL milk sample, and shaking at 25 °C for 2 h (*15*). Following incubation, the mixture was transferred to centrifuge tubes, and centrifugated for 15 min at 3000 rpm (swing out head 34121-613, MSE Scientific Instruments, U.K.). The supernatant was then carefuly filtered (0.2 *μ*m filter) into Eppendorf tubes and analyzed by HPLC.

Loss of isoflavones and the formation of decomposition products were detected by HPLC (HP 1100) equipped with a diode-array detector, and controlled by the ChemStation software package (Hewlett-Packard, Wilmington, DE). HPLC analysis was carried out on a reversed-phase C_{18} column (25 cm × 4.6 mm SupelcoSil column, Supelco). Samples were eluted at a flow rate of 1 mL/min by using a gradient of 25% methanol (A) in citrate solution (pH 3.5) (B) at time 0 up to 50% B at 20 min and an isocratic eluant to the end of the program (overall length of 38 min). Eluted isoflavones were detected at 262 nm. Isoflavones were quantified by external standards of genistein, genistin, and daidzein of Sigma Chemical Co. (St. Louis, MO), and daidzin, 6″-*O*-acetylgenistin, and 6″-*O*-acetyldaidzin from LC Laboratories (Woburn, MA).

Data Analysis. The initial isoflavone concentration was determined by using 4−8 replicates, and all storage experiments were perfomed in triplicates, extracting from each replicate. Unless stated otherwise, the results are presented as the means ± the standard deviation (SD). Statistical analysis was performed by using the data analysis tool pack of Mirosoft Excel 2000 software.

RESULTS AND DISCUSSION

The overall goal of our study was to evaluate the stability of isoflavones in soy milk. Initially, the concentrations of various isoflavone derivatives in the tested soy milk were determined. Commercial soy milk samples were analyzed for isoflavones, and three major isoflavones were detected: genistin, 141.7 mg/L ± 11.5 SD; daidzin, 29.1 mg/L ± 6.7 SD; 6″-*O*-acetydaidzein, 21.0 mg/L ± 3.1 SD. The concentrations of other isoflavones were (genistein) 5.36 mg/L ± 0.16 SD, (daidzein) 2.60 mg/L ± 1.27 SD, and (6″-*O*-acetylgenistin) 3.29 mg/L ± 0.90 SD. This profile of genistein and daidzein derivatives is in agreement with previous reports, showing that the genistin and daidzin concentrations in soy milk range from 24 and 12 mg/L to 130 and 94 mg/L, respectively (*8*−*9*, *11*, *15*−*18*). Due to its relatively high concentration in the soy milk samples, most of the kinetic study was performed on the loss of genistin.

Kinetics of Genistin Loss at Elevated and Ambient Temperatures. The kinetics of genistin loss in soy milk was determined by following its concentration in soy milk samples stored at various temperatures. Soy milk samples were initially incubated at elevated temperatures, ranging from 70 to 90 °C, for the purpose of accelerated studies of genistin stability. The decrease in genistin concentration appeared to be of first-order kinetics, as shown in **Figure 1** for the degradation of genistin at 70 °C. Indeed, genistin degradation at elevated temperatures followed first-order kinetics, as shown in **Figure 2**. A similar experimental setup for ambient temperature ranging from 15 to 37 °C revealed the same degradation pattern. Assuming first-order kinetics for genistin degradation in all incubation temperatures, rate constants were calculated (**Table 1**). Reaction rate constants at the higher temperature range were found to be 2−3 orders of magnitude higher than the rates at ambient temperatures. The poor correlation coefficients for the rate constants are due to the combination of moderate changes in genistin content and high variability in the data. However, the regression of $\ln(C/C_0)$ vs time that was used to calculate the reaction rates for 25 and 37 °C was significant (**Table 1**). In light of these observations, while indicating a true decrease in the isoflavone concentration, the values of the rate constants given in **Table 1** should be used carefuly.

The results obtained in the kinetic study are in agreement with previous works on isoflavone stability. Grun et al. (*19*)

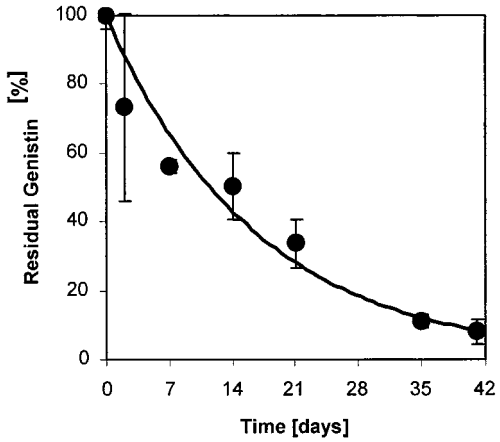

Figure 1. Genistin loss in soy milk stored at 70 °C. Milk samples (5 mL) were stored at 70 °C, and the isoflavones were extracted with 80% methanol and analyzed by HPLC.

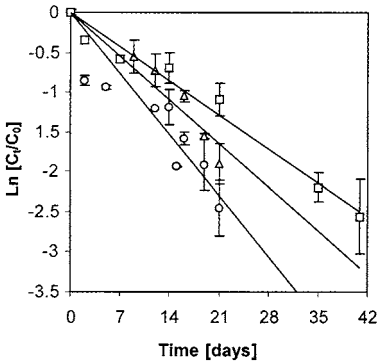

Figure 2. Degradation of genistin in soy milk stored in elevated temperatures: 70 (□), 80 (△), and 90 (○) °C.

Table 1. Kinetic Constants for Genistin Degradation in Soy Milk

temp (°C)	rate constant (10^3 days^{-1})	r^2	p^a
15	0.437	0.025	0.095
25	1.111	0.392	<0.001
37	3.871	0.433	<0.001
70	61.055	0.833	<0.001
80	77.845	0.919	<0.001
90	109.162	0.944	<0.001

[a] The significance of the linear regression of $\ln(C/C_0)$ vs time.

have shown that also during thermal treatment of tofu, there is a decrease in the total isoflavone content. Assuming first-order kinetics, isoflavone degradation during 40 min at 80, 90, and 100 °C revealed kinetic constants of 5−13 days^{-1}. These rate constants are 3 orders of magnitude higher than the values found in the present study for genistin in soy milk. This difference can be explained by the observation of Grun et al. (*19*), showing that after 40 min of thermal treament genistein derivatives are only slightly affected, while most of the isoflavone decomposition is due to loss of free daidzein, which may be more labile to thermal tretments than genistein. In the study of Davies et al. (*20*) using model genistein solutions, the genistein concentration decreased rapidly and reached 50% of the initial concentration after less than a week of incubation at 60 °C. The reaction kinetics was analyzed assuming zero-order kinetics; however, all model systems had pH 9, higher than that of soy milk.

Temperature Dependence of Genistin Loss Rate. The temperature dependence of genistin degradation rates in soy milk

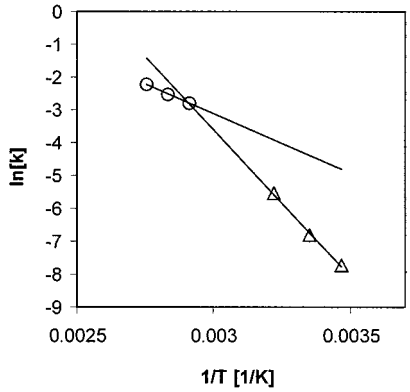

Figure 3. Arrhenius plot of genistin loss rate constants in soy milk stored at ambient (△) (15, 25, and 37 °C) and elevated (○) temperatures (70, 80, and 90 °C).

was calculated by using the Arrhenius plot (**Figure 3**). The calculated activation energies for genistin loss at elevated and ambient temperatures were 17.6 ± 0.9 and 7.2 ± 0.8 kcal/mol, respectively (average ± standard error). These low activation energy values may indicate some type of oxidation reaction. In addition, the difference in the apparent activation energies at high and low temperature suggests that a number of reactions take place at the same time. In this case, one reaction may become dominant at elevated tempeatures, and thus change the apparent activation energy of the measured degradation rate. Meta analysis of the data for total isoflavone loss during themal treatment of tofu results in an activation energy for daidzein degradation of 19.5 kcal/mol (*19*). This higher sensitivity of daidzein loss rate to temperature is also expressed by their results showing that its loss rate was the highest among isoflavones in tofu.

Degradation of isoflavones during thermal treatments was also demonstrated for extruded foods. Mahungu et al. (*21*) showed that extruded soy protein samples had lower isoflavone levels than preextruded samples. Overall losses of 22%, 24%, and 26% were observed when the mixture was extruded with barrel temperatures of 110, 130, and 150 °C, respectively. In this study too, the loss of daidzein series compounds was higher than the loss of genisteins. However, the concentration of both daidzein and genistein series decreased following extrusion. They suggested that it could be due to the destruction of isoflavones or to the difference in extractability of the compounds.

Stability of Daidzein Derivatives. During the HPLC analysis of the genistin content in soy milk, an increase of 6″-*O*-acetyldaidzin was detected in soy milk stored at elevated temperatures, as early as 24 h following the incubation of the milk at 90 °C. Following the initial increase in its concentration, the 6″-*O*-acetyldaidzin concentration decreased at what seems to be zero-order kinetics until it was paractically lost after 12 days of incubation (**Figure 4**). This observation concurs with earlier research showing an increased 6″-*O*-acetyldaidzin concentration following thermal treatment (*11*). It should be noted that, in the ambient temperature storage experiments, this increase was not detected. Analysis of the daidzin content in the soy milk showed that its concentration did not change significantly, and it is possible that this pseudoconstant concentration is a result of a combination of daidzin degradation and acetyldaidzin deacetylation to form new daidzin. It should be noted that the daidzin concentration in soy milk did not change significantly during storage in the low temperature range of 15−37 °C.

Evidence for one potential route of isoflavone degradation in composite solutions was brought by Davies et al. (*20*). Their

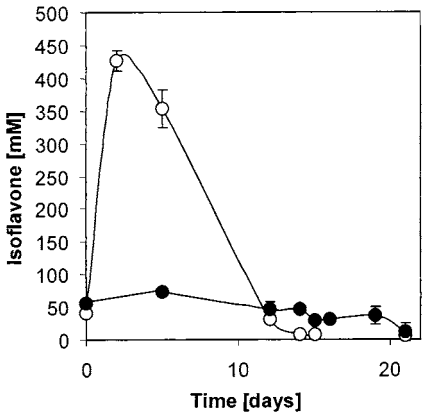

Figure 4. Change of daidzin (●) and acetyldaidzin (○) concentrations during soy milk storage at 90 °C.

study supported the notion that isoflavones can react in Maillard-type reactions in model systems containing either soy protein or whey proteins. However, their results showed that the genistein concentration decreases also in the absence of amino acids and proteins. It should be noted that Maillard reaction products are known to be potential carcinogens, and therefore, isoflavones participating in this reaction may produce undesirable products. Indeed, Singletary et al. (*10*) found that extrusion of soy−corn mixtures changes the antiproliferative activity of the isoflavone extract derived from these mixtures. The decrease in their antiproliferative activity was approximately 34−94%.

In summary, the data indicate that genistin in soy milk is labile to degradation during storage. Although the loss rate was low at ambient temperatures, one should consider the potential loss of genistin when estimating the potential shelf life of soy milk products. In addition, new intermediates produced during the accelerated kinetic study at elevated temperatures should draw our attention to the potential changes in the bioactivity of the isoflavone mixture in soy products stored for extended periods, as was the case for Gallaher et al. (*22*). Further kinetic studies and identification of these intermediates may shed light on the changes in the biological potential of isoflavones during processing and storage of soy foods.

LITERATURE CITED

(1) Clarckson, T. B.; Anthony, M. S.; Hughes, C. L., Jr. Estrogenic soybean isoflavones and chronic disease: risks and benefits. *Trends Endocrinol. Metab.* **1995**, *6*, 11−16.

(2) Adlercreutz, H. C.; Goldin, B. R.; Gorbach, S. L. Soybean phytoestrogen intake and cancer risk. *J. Nutr.* **1995**, *125*, 757S−770S.

(3) Kurzer, M. S.; Xu, X. Dietary phytoestrogens. *Annu. Rev. Nutr.* **1997**, *17*, 353−381.

(4) Anderson, J. J. B.; Garner, S. C. Phytoestrogene and bone. In *Phytoestrogens*; Adlercreutz, H., Ed. *Balliere's Clin. Endocrinol. Metab.* **1998**, *12*, 543−557.

(5) Setchell, K. D. R. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl.), 1333S−1346S.

(6) Kinoshita, E.; Sugimoto, T.; Ozawa, Y.; Aishima, T. Differentiation of soy sauce produced from whole soybeans and defatted soybeans and pattern recognition analysis of HPLC profiles. *J. Agric. Food Chem.* **1998**, *46*, 877−883.

(7) Wang, H.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666−1673.

(8) Murphy, P. A.; Song, T. T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D.; Holden, J. Isoflavone inretail and institutional soy foods. *J. Agric. Food Chem.* **1999**, *47*, 2697−2704.

(9) Wang, G.; Kuan, S. S.; Francis, O. J.; Ware, G. M.; Carman, A. S. A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *J. Agric. Food Chem.* **1990**, *38*, 185−190.

(10) Singletary, K.; Faller, J.; Li, J. Y.; Mahungu, S. Effect of extrusion on isoflavone content and antiproliferative bioactivity of soy/corn mixtures. *J. Agric. Food Chem.* **2000**, *48*, 3566−3571.

(11) Wang, H. J.; Murphy, P. A. Mass balance study of isoflavones during soybean processing. *J. Agric. Food Chem.* **1996**, *44*, 2377−2383.

(12) Coward, L.; Smith, M.; Kirk, M.; Barne, S. Chemical modification of soy foods during cooking and processing. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl.), 1486S−1491S.

(13) Farmakalidis, E.; Murphy, P. A. Isolation of 6-O-acetyldaidzein and 6-O-acetylgenistein from toasted defatted soy flakes. *J. Agric. Food Chem.* **1985**, *33*, 385−389.

(14) Kudou, S.; Fleury, Y.; Welti, D.; Magnolato, D.; Uchida, T.; Kitamura, K.; Okubo, K. Malonyl isoflavone glycosidesin soybean seeds (*Glycine max* merrill). *Agric. Biol. Chem.* **1991**, *55*, 2227−2233.

(15) Barnes, S.; Kirk, M.; Coward, L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. *J. Agric. Food Chem.* **1994**, *42*, 2466−2474.

(16) Song, T.; Barua, K.; Buseman, G.; Murphy, P. A. Soy isoflavones analysis: quality control and a new internal standard. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl.), 1474S−1479S.

(17) Fukutake, M.; Takahashi, M.; Ishida, K.; Kawamura, H.; Sugimura, T.; Wakabayashi, K. Quantification of genistein and genistin in soybeans and soybean products. *Food Chem. Toxicol.* **1996**, *34*, 457−461.

(18) Choi, Y.-S.; Lee, B.-H.; Kim, J.-H.; Kim, N.-S. Concentration of phytoestrogens in soybeans and soybean products in Korea. *J. Sci. Food Agric.* **2000**, *80*, 1709−1712.

(19) Grun, I. U.; Adhikari, K.; Li, C.; Li, Y.; Lin, B.; Zhang, J.; Fernando L. N. Changes in the profile of genistein, daidzein, and their conjugates during thermal processing of Tofu. *J. Agric. Food Chem.* **2001**, *49*, 2839−2843.

(20) Davies, C. G. A.; Netto, F. M.; Glassenap, C. M.; Gallaher, C. M.; Labuza, T. P.; Gallaher, D. D. Indication of the Maillard reaction during storage of protein isolates. *J. Agric. Food Chem.* **1998**, *46*, 2485−2489.

(21) Mahungu, S. M.; Diaz-Mercado, S.; Li, J.; Schwenk, M.; Singletary, K.; Faller, J. Stability of isoflavones during extrusion processing of corn/soy mixture. *J. Agric. Food Chem.* **1999**, *47*, 279−284.

(22) Gallaher, D. D.; Gallaher C. M.; Hoffman, R. M. Soy protein isolate and Genistein: effects on initiation and progression of colon cancer. Presented at the Second International Symposium on the Role of Soy in Preventing and Treating Chronic Disease, Brussels, Belgium, Sept 15−18, 1996.

Received for review July 2, 2002. Revised manuscript received January 15, 2003. Accepted February 6, 2003. The study was partially supported by the United States−Israel Binational Science Foundation (Research Project 2000137), and by the fund for the promotion of research at the Technion.

JF025783H