FORTIFICATION OF SOY PROTEIN WITH CHEESE WHEY PROTEIN AND THE EFFECT OF ALKALINE pH

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ABSTRACT

Replacement of soy protein with whey protein in the range of 0 to 40% increased the protein efficiency ratio (PER) from approximately 1 to up to 3. PER improvement was similar for both "heat-denatured" and "nonheated" (recovered by ultrafiltration) whey protein, and was due to a better balance of the essential amino acids in the protein mixture, as compared to that of the soy protein alone. Of special importance was the contribution of the whey protein in methionine (1.8 g/100 g protein) and in cysteine/cystine (2.8 g/100 g protein) which are the limiting amino acids of the soy protein (1.3 and 0.9 g/100 g protein, respectively). Exposure of the individual soy and whey proteins, as well as their mixture to alkaline pH, caused a loss in PER which was originated mainly by the destruction of cysteine/cystine. The influence of pH, temperature, and the time-length of the exposure to alkali on this destruction was measured at a constant concentration of 14% protein. At pH 12.5 and room temperature, the damage was small even after 15 min; damage increased with temperature, and at 65°C, 0.4–0.6 g/100 g protein of these amino acids were destroyed in the first 90 sec, causing a 10% drop in the PER. The critical pH for both proteins was higher than 11, since at pH 11 only a very slight damage was observed under similar conditions. Destruction of cysteine/cystine yields mainly dehydroalanine, and this compound reacts readily with several amino acids. For example, we have found that after 2 hr at pH 12.5, 65°C, approximately half of the decomposed cysteine/cystine had reacted with the ϵ-amino group of lysine to form lysinoalanine (LAL). After 90 sec at pH 12.5 and 65°C, the amounts of LAL found in the samples were approximately 0.2 g/100 g of soy protein, and 0.5 g/100 g of whey protein. These findings should provide a valuable guideline for further development and improvement of the spinning technology.

The need for more efficient exploitation of the soy protein and better consumer acceptability of soy products has led to the development of several new technologies; those developed for manufacturing textured foods are of major importance. One of these technologies is acid spinning, which permits the manufacture of fibrous protein structures resembling meat fibers. The process consists of injecting an alkaline soy protein solution (the dope) through a punched plate (the spinneret) into an acid bath. Upon contact with the acid, the pH of the dope is reduced almost instantly to the isoelectric point of the protein, which coagulates and forms the fibers. The fibers are then washed and used in the manufacture of meat analogues.

From the nutritional point of view, the use of vegetable protein as well as the spinning process in itself may raise two problems. The first problem is the low nutritional value of vegetable proteins due to their amino acid imbalance. For example, commercial soy isolates have a protein efficiency ratio (PER) of 1.0 to 1.8 (1), relative to 2.5 for casein, whereas meat proteins in general have a PER above 2.5. This low PER is due essentially to a deficiency in the sulfur-containing amino acids in soy protein (2). This problem may be corrected by supplementing the protein with D,L-methionine (1), with the dipeptide D,L-methionyl-D,L

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methionine (3), or with acetylated derivatives of L-methionine and L-cysteine (4–6). On the other hand, mixtures of soy protein and proteins rich in sulfur amino acids, as for example cheese whey protein, have a relatively high PER (approximately 3.0) (7). Thus, as in the case of supplemented soy protein fibers, soy/whey protein fibers should have a higher PER than nonsupplemented fibers.

The second problem is that exposure of the proteins to the alkaline pH required for spinning may cause the destruction of some amino acid residues, with the consequent loss in nutritional value. The most sensitive amino acids to alkali are cysteine and cystine (8): they undergo desulfuration to yield dehydroalanine (9,10) which, in turn, may react with the ϵ-amino group of free or bound lysine to yield lysinoalanine (LAL) (11), with free or bound ornithine (usually not present in food proteins) to yield ornithinoalanine (12), with free or bound cysteine to yield lanthionine (13), or with water to yield either D,L-serine or pyruvic amide (9). Most of these reactions lead to the formation of new nonpeptide bridges between amino acid residues which are stable to hydrolysis by digestive proteases and may reduce the utilization of the protein (14). O-substituted serine (phosphorylated or glycosylated) undergoes similar reactions (11). Other reactions that may occur at alkaline pH are the deguanidination of arginine to yield ornithine and the isomerization of isoleucine on carbon 3, to yield alloisoleucine (15). However, these reactions are slower and thus less important in the spinning process, and have much less influence on the nutritive value than the decomposition of cysteine/cystine.

The destruction of cysteine and cystine at alkaline pH has been measured by several researchers (8–11, 14–16). Exposure of soy isolates to pH 12.2 at 40°C for periods of time between 1 and 8 hr may destroy over 50% of their cysteine/cystine content (14). Furthermore, 0.1*N* sodium hydroxide at 60°C destroyed in 8 hr all the cysteine/cystine of a nonedible soy isolate (17). Similarly, all the cysteine/cystine disappeared in a sunflower isolate treated 1 hr at 55°C and pH 11.5 (15). Parallel to these observations, losses in nutritional value (14) and the formation of up to 2.6 g of LAL/100 g protein have been measured (17). Obviously, exposure of proteins to alkali during very short periods of time (below 10 min), as is the case during the manufacture of spun fibers, will cause much less protein damage than that cited above. However, losses in cysteine/cystine as well as formation of measurable amounts of LAL during spinning have been reported (18,19).

Bohak (11) and Freimuth *et al.* (20) studied the formation of LAL in diluted solutions of purified proteins (ribonuclease, phosvitin, BSA, papain, lysozyme, α-chymotrypsinogen, α-lactalbumin, β-lactoglobulin, and casein fractions), when exposed to strong alkaline treatment. They suggest that LAL is formed in proteins as a function of their content in the amino acid sequences Lys-Cys (or Lys-Ser), and Cys-Lys (or Ser-Lys). More recently, LAL has been detected at the ppm level in a number of heated food products, even without any alkaline treatment (21). Further studies are needed to establish the differences in susceptibility to chemical modifications at elevated pH of different food proteins, under specified technological conditions.

The purposes of our research were: 1) to determine quantitatively the effect of the addition of cheese whey protein to soy protein on the nutritional value of spun fibers made from these raw materials, and 2) to measure the loss in cysteine and in cystine of these proteins when subjected to alkaline treatments, as a

function of pH, temperature, and time. The information gained should be of value for improving the present stage of the spinning technology.

MATERIALS AND METHODS

Raw Materials

All our tests on soy protein were made with a commercial soy isolate (Promine R from Central Soya). The whey protein was prepared in our pilot plant, starting from sweet cheese whey obtained from a local supplier. Two samples of whey protein were manufactured: sample A, recovered from the whey by heat precipitation at pH 4.5 (100° C, 20 min), and sample B, recovered from whey by ultrafiltration using an industrial module manufactured by the Danish Sugar Factories (DDS) equipped with type 600 membranes. Our raw materials were free of detectable LAL.

Laboratory Samples

The pH of the protein solutions was increased by adding 10*N* sodium hydroxide. The amount of sodium hydroxide necessary to attain a given pH was predetermined by making a titration curve at room temperature. The pH value was checked again at the beginning and at the end of each experiment (at room temperature).

The 14% protein solutions were equilibrated in a water bath for 15 min at the selected experimental temperature, and the predetermined amounts of sodium hydroxide were quickly added under strong mechanical stirring; 10-ml samples were withdrawn with a syringe from these solutions after 0.75, 1.5, 3, and 15 min, and transferred into a centrifuge tube containing the amount of 1*N* HCl necessary for reducing the pH below 7. Control samples were withdrawn from each protein solution before the addition of NaOH. The samples were then adjusted to pH 4.5 with 1*N* HCl and centrifuged. The sediments were separated and lyophilized. All the analytical work was done on these lyophilized samples.

Analysis

Nitrogen analyses were made on the Technicon AutoAnalyzer. Protein contents were calculated by multiplying the total nitrogen by 6.25. Cysteine/cystine was determined by the method of Gaitonde (22) adapted for manual operation, with the following modification: Hydrolysis was conducted at 120° C during 24 hr, in a glass ampoule flushed with nitrogen gas and sealed under vacuum, with the equivalent of about 500 μg of cystine in 5 ml 6*N* HCl saturated with nitrogen gas. Results are expressed in g of cysteine/100 g of protein. Gaitonde's method is based on the photometric determination of the reaction product between ninhydrin and cysteine (reduced by dithiothreitol) in a strongly acidic medium. Control analysis showed that the results were systematically about 25% lower than those obtained by performic oxidation of cysteine/cystine and measurement of the resulting cysteic acid in the amino acid analyzer. No explanation was found for this discrepancy, but reproducibility was satisfactory (±10%). This method was selected because of its rapidity and convenience for the analysis of large series of samples. LAL was determined in a few selected samples with the Beckman Multichrom amino acid analyzer on the short column used for the basic amino acids. A standard of LAL was synthesized

for this purpose. Since the molecular-weight ratio of cysteine/LAL is 0.52, 1 g of cysteine may yield a maximum of approximately 2 g of LAL.

Protein Efficiency Ratio (PER)

The 500-g protein samples were treated in the pilot plant at pH 12.5 in a continuous system, with a residence time of 1.5 min at 65°C, followed by approximately 1 min at room temperature before neutralization. The PERs were determined by the following procedure: groups of 6 Sprague-Dawley male rats (age 23 days, weight 50 to 60 g) were housed individually in screen-bottom cages and fed the test protein at the 10% level in the diet. The diet also contained 10% oil, 25% sucrose, 3% minerals, vitamins, and corn starch to 100%. A reference group was fed Hammersten casein in the diet, instead of the test protein. Growth curves were drawn for 3 weeks, and the PERs were calculated by dividing the mean weight gain by the protein consumption. The average PER for the casein reference group was 3.2.

RESULTS AND DISCUSSION

Figure 1 shows the effect of soy protein replacement with cheese whey protein on the nutritional value of the protein (PER) before and after the alkaline treatment in the pilot plant. Results were the same for the two types of whey

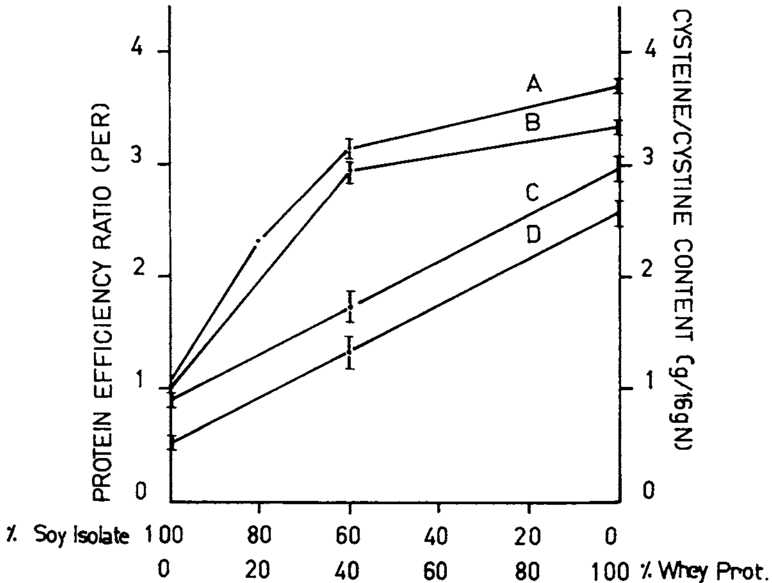

Fig. 1. Improvement of the protein efficiency ratio (PER) and cysteine/cystine content of soy protein by addition of whey protein. A = PER before alkaline treatment; B = PER after alkaline treatment; C = cysteine/cystine before alkaline treatment; and D = cysteine/cystine after alkaline treatment. Vertical bars represent standard deviations. Standard PER of casein = 3.2.

protein used. Replacement in the range from 20 to 40% improved the PER value enormously: the PERs were 2.35 ± 0.08 and 3.09 ± 0.05 for 20 and 40% replacement, respectively. The PER of the soy isolate we used was 1.12 ± 0.17. The steep slope of the PER curve below 40% replacement was due to the contribution of the whey protein in sulfur amino acids, and eventually tryptophan, up to the optimum level. The two straight lines below the PER curves represent the increase in cysteine/cystine, as the content of whey protein in the mixture is increased. It is evident from these curves that the alkaline treatment caused a significant loss in cysteine/cystine of about 0.4 g/100 g protein, with a subsequent small loss in PER, significant only at the 10% level. Thus, laboratory experiments were made in order to determine quantitatively the effects of pH, temperature, and time on the destruction of these amino acids.

Figure 2 shows the effect of time on the destruction of cysteine/cystine at pH 12.5, 65° C. The shape of the destruction curves for the soy and the two whey proteins was similar: cysteine/cystine destruction was fast at the beginning of exposure to alkali and then tended to level off between 50 and 70% destruction. The percentage of destruction was higher in the soy than in the whey protein. Obviously, in absolute amounts the reverse was true, since untreated whey protein contains three to four times as much cysteine/cystine as soy protein.

The difference observed in the amino acid destruction rate between the soy and the whey protein may be due to different amounts of Cys-Lys and Lys-Cys bonds in the protein chains, as stated by Bohak (11) and Freimuth *et al.* (20).

Figure 3 shows the effect of pH on the destruction of cysteine/cystine at 65° C.

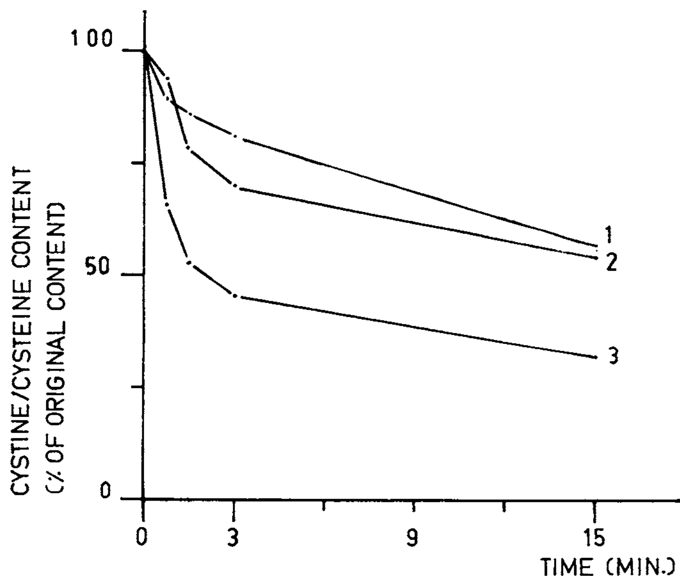

Fig. 2. Effect of time on the destruction of cysteine/cystine by alkali (pH 12.5, temperature 65° C). 1 = Whey protein, sample B (original cysteine/cystine: 2.9 g/16 g N); 2 = whey protein, sample A (original cysteine/cystine: 3.0 g/16 g N); and 3 = soy isolate (original cysteine/cystine: 0.9 g/16 g N).

Amino acid destruction at 65° C was very small below pH 11, even after 15 min of alkaline treatment, whereas above pH 12 damage became important, even after 3 min. After 1.5 min at 65° C, the LAL contents of the samples at pH 12 and 12.5 were, respectively, 0.43 and 0.54 g/100 g of whey protein (recovered by ultrafiltration), and 0.1 and 0.18 g/100 g of soy protein.

Temperature drastically influenced the rate of destruction of cysteine/cystine at pH 12.5, as illustrated in Fig. 4. At 25° C, destruction was relatively small. Destruction rate increased with temperature: at 75° C, 40% of the cysteine/cystine of the soy protein was destroyed in the first 45 sec, and up to 65% was destroyed in 15 min. The corresponding values for the whey protein (recovered by ultrafiltration) were 15 and 50%. Formation of LAL was also favored by higher temperatures: upon increasing the temperature from 55° to 65° C, the amount of this compound found in the samples increased from 0.10 to 0.18 g/100 g of soy protein, and from 0.45 to 0.54 g/100 g of whey protein.

In conclusion, this research shows that if the experimental conditions in a pilot plant continuous operation for the manufacture of soy protein fibers are 65° C, pH 12.5, 1.5 min, destruction of cysteine/cystine is approximately 40% of the total content of this amino acid. Doubling the exposure time to alkali increases

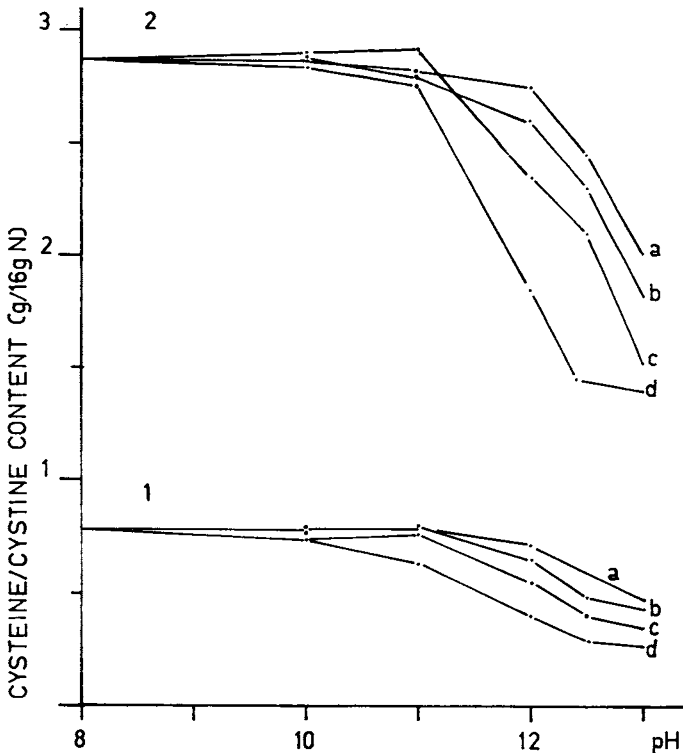

Fig. 3. Effect of pH on the destruction of cysteine/cystine in 1) soy protein and 2) whey proteins, sample B; temperature 65° C. Exposure time: a = 0.75 min; b = 1.5 min; c = 3 min; and d = 15 min.

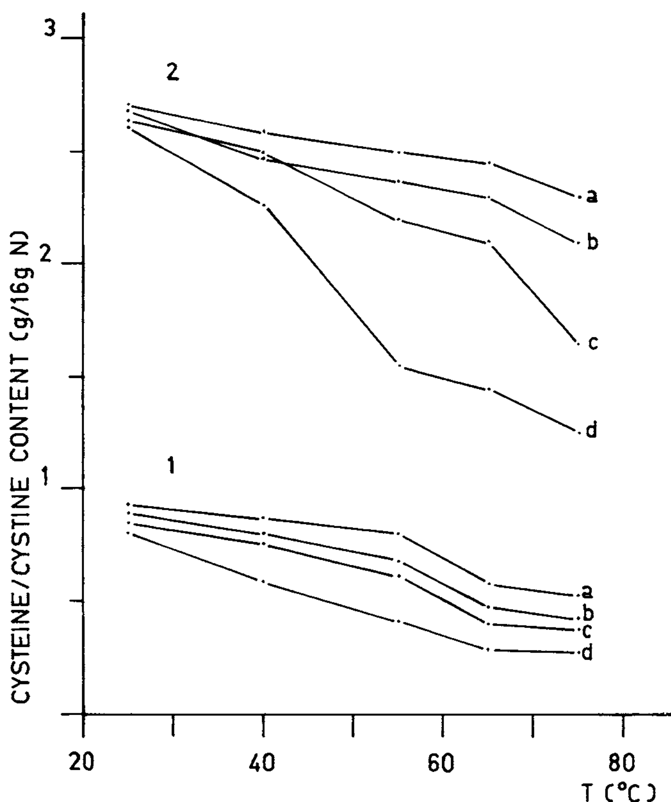

Fig. 4. Effect of temperature on the destruction of cysteine/cystine of 1) soy protein and 2) whey protein, sample B, at pH 12.5. Exposure time: a = 0.75 min; b = 1.5 min; c = 3 min; and d = 15 min.

the loss to over 50%; reducing it to 45 sec (half the time) reduces the loss to 25%. Reduction of the temperature from 65° to 55°C also decreases the amino acid loss to 25%.

Our results suggest that simultaneous reduction of temperature from 65° to 55°C, time from 90 to 45 sec, and pH from 12.5 to 12.0 will decrease losses by over one-half, the maximum loss then being approximately 15% of the total content of cysteine/cystine, with less than 0.05% LAL being formed.

Similar conclusions may be drawn for the whey protein. However, although a lower proportion of cysteine/cystine will be destroyed, on absolute values the amino acid destruction will be higher, since this protein is richer in cysteine/cystine than the soy protein. At pH 12.0, 55°C for 45 sec, less than 0.25% LAL will be formed. Thus, under similar conditions, a mixture composed of 25% whey protein and 75% soy protein may be expected to yield less than 0.1% LAL. Incorporation of the protein mixture at a 15–20% level in the diet will therefore result in less than 100 ppm of LAL in the diet. These figures compare favorably with those published by Sternberg *et al.* (21). On the other hand, the

PER of the spun fibers of the mixture will be above 2, as compared to 1.0 for our fibers of soy protein alone, and 1.1 for the isolate used.

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Literature Cited

1. MATTIL, K. F. Composition, nutritional, and functional properties, and quality criteria of soy protein concentrates and soy protein isolates. J. Amer. Oil Chem. Soc. 51: 81A (1974).
2. SMITH, A. K., and CIRCLE, S. J. Soybeans: Chemistry and technology. Avi Pub. Co.: Westport, Conn. (1972).
3. Deutsche Gold- und Silberscheidenanstalt. Lebensm. Futterzusatzstoff, German Patent 2'251'877 (1974).
4. AMOS, H. E., SCHELLING, G. T., DIGENIS, G. A., SWINTOSKY, J. E., LITTLE, C. O., and MITCHELL, J. E., Jr. Methionine replacement value of N-acetylmethionine and homocysteine-thiolactone hydrochloride for growing rats. J. Nutr. 105: 577 (1975).
5. BOGGS, R. W., ROTRUCK, J. T., and DAMICO, R. A. Acetyl-methionine as a source of methionine for the rat. J. Nutr. 105: 326 (1975).
6. PROCTER & GAMBLE. Ergänzung von Nahrungsmitteln mit N-acyl-derivaten schwefelhaltiger L-Aminosäuren. German Patent 2'326'444 (1973).
7. LEFAIVRE, J., TREMBLAY, L., JULIEN, J. P., and BRISSON, G. J. Rôle de complémentarité des protéines solubles du lait pour les protéines de la fève soya. Can. Inst. Food Sci. Technol. J. 5: 87 (1972).
8. ZAHN, H., and GOLSCH, E. Zersetzung von Cystin, Cystein, Cystindihydanthoin, Lanthionin and Lanthionindihydanthoin in wässrigen Lösungen. Z. Physiol. Chem. 330: 38 (1962).
9. DE MARCO, C., COLETTA, M., and CAVALLINI, D. Cystine cleavage in alkaline medium. Arch. Biochem. Biophys. 100: 51 (1963).
10. GAWRON, O., and ODSTRCHEL, G. Kinetic studies on the alkaline decomposition of cystine derivates and peptides. J. Amer. Chem. Soc. 89: 3263 (1967).
11. BOHAK, Z. N-ε(D,L-2-amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. J. Biol. Chem. 239: 2878 (1964).
12. ZIEGLER, K., MELCHERT, I., and LUERKEN, C. Nδ-(2-amino-2-carboxyethyl)-ornithine, a new amino acid from alkali treated proteins. Nature 214: 404 (1967).
13. HORN, M. J., JONES, D. B., and RINGEL, S. J. Isolation of a new sulphur-containing amino acid (lanthionine) from sodium carbonate-treated wool. J. Biol. Chem. 138: 141 (1941).
14. DE GROOT, A. P., and SLUMP, P. Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. J. Nutr. 98: 45 (1969).
15. PROVANSAL, M. Contribution à l'étude des modifications chimiques des protéines sous l'influence des traitements technologiques alimentaires. Effets des traitements alcalins sur la valeur nutritionnelle d'aliments protéiques. Ph.D. Thesis, Montpellier, France (1974).
16. ISHINO, K., and OKAMOTO, S. Molecular interactions in alkali-denatured soybean proteins. Cereal Chem. 52: 9 (1975).
17. WOODARD, J. C., and SHORT, D. D. Toxicity of alkali-treated soy protein in rats. J. Nutr. 103: 569 (1973).
18. VAN BEEK, L., FERON, V. J., and DE GROOT, A. P. Nutritional effect of alkali-treated soy protein in rats. J. Nutr. 104: 1630 (1974).
19. BRESSANI, R., VITERI, F., ELIAZ, L. G., DE ZAGHI, S., ALVARADO, J., and ODELL, A. D. Protein quality of a soybean protein textured food in experimental animals and children. J. Nutr. 93: 349 (1967).
20. FREIMUTH, U., SCHLEGEL, B., GAHNER, E., and NÖTZOLD, H. Die Bildung von Lysinoalanin in alkalibehandelten Milchproteinen. Nahrung 18: K5 (1974).
21. STERNBERG, M., KIM, C. Y., and SCHWENDE, F. J. Lysinoalanine: presence in foods and food ingredients. Science 190: 992 (1975).
22. GAITONDE, M. K. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. Biochem. J. 104: 627 (1967).

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