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Removal of Nucleic Acids from Yeast Nucleoprotein Complexes by Sulfitolysis

Srinivasan Damodaran

A simple method for removal of nucleic acids from yeast nucleoprotein complexes by sulfitolysis is described. Treatment of yeast nucleoproteins with sodium sulfite followed by sodium tetrathionate caused destabilization and dissociation of nucleoprotein complexes. Subsequent precipitation of proteins at pH 4.2 resulted in a protein preparation with low levels of nucleic acids. A good correlation between the extent of nucleic acid removal and the disulfide content of the sample was also observed. However, when the nucleoproteins were treated with either sodium sulfite or sodium tetrathionate only, there was no appreciable removal of nucleic acids. Further, the efficiency of nucleic acid removal by sulfitolysis also depended on the initial nucleoprotein concentration. The mechanism of dissociation of nucleoproteins by sulfitolysis and the advantages of sulfitolysis over those of other chemical modification procedures to reduce nucleic acid content in single cell proteins are also discussed.

INTRODUCTION

One of the major problems limiting the exploitation of proteins from microbial sources is the high level of nucleic acid contamination in these protein preparations (Sinskey and Tannenbaum, 1975; Vananuvat and Kinsella, 1975; Lipinskey and Litchfield, 1974). From nutritional point of view, consumption of high levels of nucleic acids in the diet (above 2 g/day) causes disorders such as urecemia, gout, and kidney stone formation (Miller, 1968; Waslein et al., 1970). Hence, in order to develop single-cell proteins for human nutrition, it is imperative to develop methodologies to reduce the nucleic acid content of the isolated protein to a safe level.

There are several methods currently available for the removal of nucleic acids from single-cell proteins (Newell et al., 1975a,b; Robbins et al., 1975; Shetty and Kinsella, 1979). However, most of these methods have major disadvantages in terms of functional quality and nutritional safety of the isolated proteins. For example, alkaline hydrolysis of nucleic acids at elevated temperatures (Newell et al., 1975b) not only results in thermal denaturation and impairment of functional properties of the protein but also causes destruction of essential amino acids and formation of potentially toxic compound known as lysinoalanine (Shetty and Kinsella, 1980). Similarly, enzymatic hydrolysis of nucleic acids results in concomitant degradation of proteins by endogenous proteases (Lindbloom, 1977). Chemical modification of yeast nucleoproteins with acyl anhydrides has been shown to decrease the nucleic acid level (Shetty and Kinsella, 1979). However, such modifications impair bioavailability of lysine. Furthermore, the biological safety of isopeptides, e.g., succinyllysine is not known. Recently, a simple and safe method using chaotropic salt treatment to reduce the nucleic acid contents in yeast protein isolates has been described (Damodaran and Kinsella, 1983a,b). However, the practicality of this approach needs further studies.

In our continuing effort to develop simple methods for the isolation of yeast proteins with low levels of nucleic acids, we studied the effect of sulfitolysis on the dissociation of yeast nucleoprotein complexes. The basic principle involved in this approach is that conversion of sulfhydryl and disulfide groups in nucleoproteins to *S*-sulfonate derivative would cause conformational changes and also increase the electronegativity of the protein

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components in nucleoproteins. These in turn may cause destabilization and dissociation of yeast nucleoprotein complexes.

EXPERIMENTAL SECTION

Materials. Sodium tetrathionate and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Nitro-5-thiosulfobenzoate (NTSB) was synthesized from DTNB according to the method described by Thannhauser et al. (1984). The concentration of NTSB in the stock solution was about 50 mM. This stock solution was stored in aliquots at −20 °C. All other chemicals used in this study were of reagent grade.

Yeast Nucleoprotein Isolate. Brewer's yeast (*Saccharomyces carlbergensis*) was obtained from a local brewery. The yeast cells were washed three times with cold distilled water and disrupted in a french press. After disruption, the broken cells were suspended in cold water at 5 °C and adjusted to pH 8.5 in order to solubilize and extract the nucleoproteins. The solution was then centrifuged at 15000*g* for 30 min at 5 °C to remove the cell wall and other insoluble materials. The nucleoproteins were then recovered from the supernatant by adjusting to pH 4.2 and centrifuging at 15000*g* for 30 min. The nucleoprotein precipitate was then redissolved in water at pH 8.5 and lyophilized. The yeast protein isolates thus prepared contained about 20–25% nucleic acids on weight basis.

Kinetic Assay for Reduction of Disulfide Bonds by Sulfite. The kinetics of reduction of disulfide bonds by sodium sulfite under nondenaturing conditions was studied by using the NTSB method as described elsewhere (Damodaran, 1985; Thannhauser, et al., 1984). In a typical experiment, to 1 mL of nucleoprotein solution (1.75%) in 0.1 M Tris-HCl buffer, pH 8.5, were added 0.10 mL of 50 times diluted NTSB stock solution and an appropriate volume of 2 M Na_2SO_3. The total volume was adjusted to 1.3 mL with the buffer. The sample was immediately placed in a Beckman Model 25 double-beam spectrophotometer. The rate of development of absorbance at 412 nm was monitored continuously against a blank containing no protein.

Sulfitolysis. In order to cleave the disulfide bonds in yeast nucleoproteins and convert the liberated sulfhydryl groups to *S*-sulfonate derivatives, the following approach was employed: To an aliquot of yeast nucleoprotein solution (2%, w/v) was added a known amount of sodium sulfite stock solution (2 M). The solution was made to a known volume. The solution was mixed well and incubated at room temperature for 10 min. During this treatment the disulfide bonds in nucleoproteins were cleaved by sodium sulfite according to the reaction (1) (Cole, 1967). At the end of 10-min incubation, an aliquot

$$RSSR' + SO_3^{2-} \rightleftharpoons RS^- + R'SSO_3^- \quad (1)$$

of 0.5 M sodium tetrathionate stock solution was added and the volume was adjusted to a known volume. The above solution was mixed well and incubated at room temperature for another 10 min. During this treatment the free sulfhydryl groups liberated from reaction 1 were oxidized by sodium tetrathionate to disulfides, according to reaction 2 (Bailey and Cole, 1959). The final concentration of nucleoprotein in all cases was 1%. At the end of the incubation period, the solution was adjusted to pH 4.2 in order to precipitate proteins. The precipitate was centrifuged, washed with water at pH 4.2, and then dissolved in water at pH 8.5. A control was run at identical conditions but with no sulfite and tetrathionate treatment. The protein, RNA, and disulfide contents of the samples were determined.

$$RSSR' + SO_3^{2-} \rightleftharpoons RS^- + R'SSO_3^- \quad (2)$$

(tetrathionate)

In another approach, in order to determine the effect of repeated reduction–oxidation cycles on the efficiency of dissociation of yeast nucleoprotein complexes, the following method was used (Bailey and Cole, 1959): To 4-mL aliquots of yeast protein solution (1%) was added 0.25 mL of 1 M sodium sulfite. The solution was incubated at room temperature for 10 min. After the incubation period, 0.2 mL of 0.5 M sodium tetrathionate was added and incubated for another 10 min. The above sodium sulfite and sodium tetrathionate treatment cycles were repeated. At the end of each cycle the protein solutions were adjusted to pH 4.2. The precipitated proteins were centrifuged, washed with water at pH 4.2, and redissolved in water at pH 8.5. Protein, RNA, and disulfide contents of the samples were determined. Unless stated otherwise, all sulfitolysis experiments were done at pH 8.5.

Sulfhydryl and Disulfide Estimation. The free sulfhydryl content was estimated by using the DTNB method (Ellman, 1959). The disulfide content was estimated by the modified 2-nitro-5-thiosulfobenzoate (NTSB) method (Thannhauser et al., 1984; Damodaran, 1985).

Protein and RNA Estimation. Protein concentration was determined by the biuret method. RNA concentration was estimated by the orcinol method (Herbert et al., 1971).

RESULTS AND DISCUSSION

Kinetics of Reduction of Disulfide Bonds. The kinetics of reduction of disulfide bonds in yeast nucleoproteins at various sodium sulfite concentrations is shown in Figure 1. The rate of increase in absorbance at 412 nm, which is due to liberation of 2-nitro-5-thiobenzoate (NTB) from NTSB (Damodaran, 1985; Thannhauser et al., 1984) was greater at higher sodium sulfite concentration. At all sodium sulfite concentrations the absorbance of the solution reached saturation values at about 10 min (Figure 1). However, the maximum absorbance at saturation kinetic conditions, i.e., 10 min, increased up to 0.2 M Na_2SO_3 (Figure 1, inset); above 0.2 M Na_2SO_3 there was no appreciable change in both the initial rate and the maximum absorbance attained by the system. This indicates that the number of disulfide bonds cleaved is a function of sulfite concentration. In order to cleave all the disulfide bonds in yeast nucleoproteins under nondenaturing conditions described here, a minimum of 0.2 M Na_2SO_3 is needed.

Effect of Sulfitolysis on the Dissociation of Yeast Nucleoproteins. The effect of sodium sulfite and sodium tetrathionate treatment on the dissociation of yeast nucleoprotein complexes is shown in Figure 2. Treatment of yeast nucleoprotein complexes first with sodium sulfite (reduction) followed by treatment with sodium tetrathionate (oxidation) caused decrease in the nucleic acid content of the protein obtained by isoelectric precipitation at pH 4.2. However, the efficiency of nucleic acid removal depended on both Na_2SO_3 and $Na_2S_4O_6$ concentrations. At any given Na_2SO_3 concentration, the efficiency of nucleic acid removal increased with the concentration of $Na_2S_4O_6$ used. However, interestingly enough, at all concentrations of Na_2SO_3 studied, the samples treated with 0.15 M $Na_2S_4O_6$ contained the same level of nucleic acid in the final preparation (Figure 2). This may indicate that for effective removal of nucleic acids from yeast nucleoprotein complexes the concentration of tetrathionate used

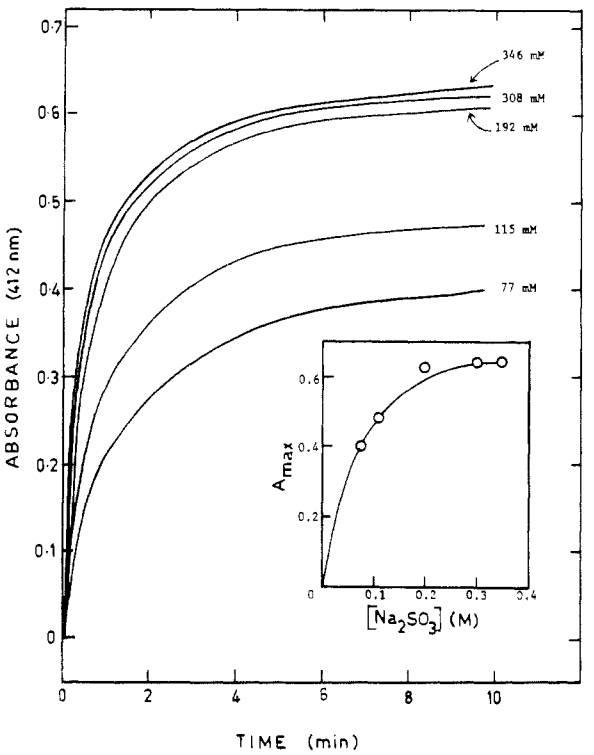

Figure 1. Effect of sodium sulfite concentration on the reduction of disulfide bonds in yeast nucleoproteins. The final concentrations of protein and NTSB in the reaction mixture were 1.35% and 0.08 mM, respectively, in 0.1 M Tris-HCl buffer, pH 8.5, containing various concentrations of sodium sulfite as indicated for each curve. The experiments were done at room temperature (24 °C). The relationship between sulfite concentration and maximum absorbance at 10 min is shown in the inset.

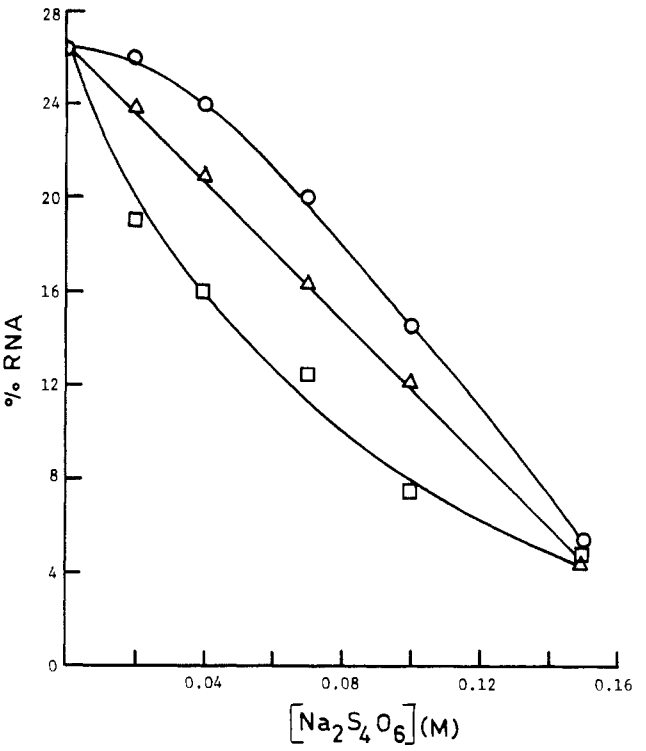

Figure 2. Effect of sodium sulfite and sodium tetrathionate treatment on the removal of nucleic acids. The curves represent the effect of 0.1 M (○), 0.2 M (△), and 0.4 M (□) Na_2SO_3. The final protein concentration in all these experiments was about 1%, and the pH was 8.5.

for reoxidation of the liberated sulfhydryl groups is more critical than the level of sulfite used for initial reduction of disulfide bonds.

In order to determine whether both reduction with Na_2SO_3 and subsequent treatment with $Na_2S_4O_6$ are essential for the dissociation of nucleoprotein complexes, the samples were treated with either Na_2SO_3 or $Na_2S_4O_6$ alone at various concentrations. After incubation for 10 min, the proteins were isoelectrically precipitated and the nucleic acid content was determined. In both cases no appreciable decrease in nucleic acid level was observed (data not shown). This suggests that, in order to induce dissociation of yeast nucleoprotein complexes, reduction of the disulfide bonds with sulfite alone is not sufficient, but reoxidation of the liberated free sulfhydryl groups with sodium tetrathionate or any other oxidizing agent is also essential. Although the reason for this is not clear, it may be speculated that the degree of structural changes in proteins caused by reduction of disulfide bonds apparently may not be sufficient enough to break the intermolecular interactions between proteins and nucleic acids. Furthermore, removal of excess sulfite during isoelectric precipitation of proteins may have facilitated re-formation of original pair of disulfide bonds according to the reaction (3). In

$$RSSO_3^- + {}^-SR' \rightleftharpoons RSSR' + SO_3^{2-} \quad (3)$$

fact, no decrease in the number of disulfide bonds was observed in samples treated with sulfite only. Such reformation of original pair of disulfide bonds will reverse the electronegativity of proteins and hence stabilize the nucleoprotein complex. However, when sodium tetrathionate is added to the sample treated with sulfite, the sulfhydryl groups liberated from the sulfite treatment (according to reaction 1) will be randomly oxidized to disulfide bonds. These disulfide bonds may in turn be reduced by excess sulfite in the system. The reduction and oxidation cycles may continue depending upon the effective concentration of sulfite and tetrathionate in the medium. Because of these reaction cycles, more and more sulfhydryl groups may be converted to *S*-sulfonate derivatives that may alter the conformation of proteins and induce dissociation of nucleoprotein complexes. The net increase in the electronegativity of sulfonated proteins may also facilitate dissociation of the complexes via electrostatic repulsion.

In order to determine whether repeated reduction–oxidation cycles would increase the efficiency of nucleic acid removal in terms of the effective concentration of sulfite and tetrathionate needed, the nucleoproteins were repeatedly reduced and oxidized using low concentrations of sulfite and tetrathionate.

The relationship between the nucleic acid content and the number of reduction–oxidation cycles treatment is shown in Figure 3. The nucleic acid content decreased with number of cycles of treatment. The total concentration of sulfite at the end of each of the four cycles studied was 59, 106, 145, and 178 mM, respectively. Similarly, the total concentration of sodium tetrathionate at the end of each of the four cycles was 22, 41, 56, and 69 mM, respectively. The disulfide content of the samples at the end of each cycle is also shown in Figure 3. For the sake of convenience the disulfide content is expressed as number of moles of disulfide bonds/100 000 g of protein. The free sulfhydryl content of the control as well as the samples, as determined by the DTNB method (Ellman, 1959), was very negligible. As shown in Figure 3, there was a good correlation between the decrease in disulfide content and the decrease in nucleic acid content as a function of the number of reduction–oxidation cycles. However, maximum reduction in disulfide content occurred within two cycles, and even after four cycles only 50% of the total

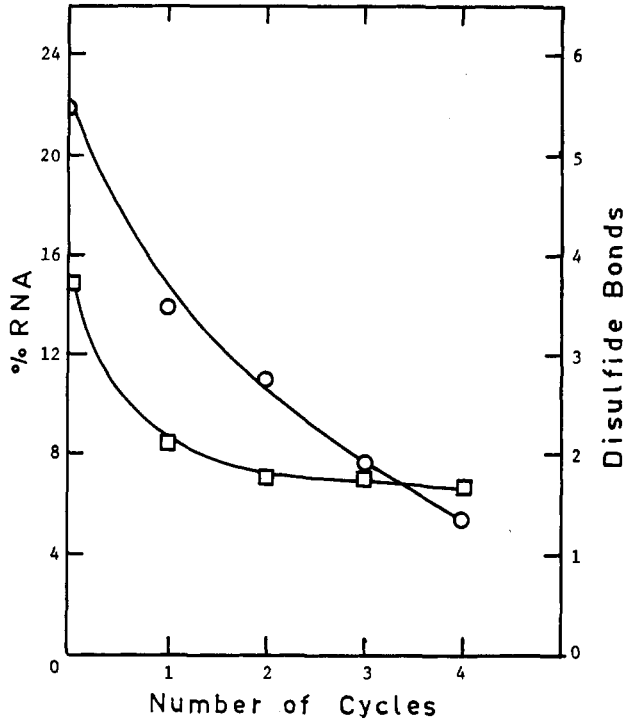

Figure 3. Effect of repeated reduction and oxidation cycles on the nucleic acid and disulfide contents of yeast protein isolate (see the text for details). ○ represents nucleic acid content. □ represents the number of disulfide bonds per 100 000 g of protein.

disulfide bonds were reduced. This may be due to the fact that some of the disulfide bonds may be buried in the interior of the protein that may not be accessible for reduction and oxidation. Nonetheless, the results suggest that complete reduction and conversion of 50% of the disulfides to *S*-sulfonate derivatives is sufficient enough to destabilize and dissociate the nucleoprotein complexes. Furthermore, it should be noted that, in order to remove about 80% nucleic acids, the repeated reduction–oxidation approach requires only 69 mM sodium tetrathionate (Figure 3) compared to 0.15 M in the case of single-cycle method (Figure 2).

Effect of Nucleoprotein Concentration. The effect of nucleoprotein concentration on the efficiency of removal of nucleic acid by sulfitolysis is shown in Figure 4. In this case the nucleoprotein samples were treated first with 0.1 M Na_2SO_3. After 10-min incubation, 0.15 M $Na_2S_4O_6$ was added and the resultant mixture incubated for another 10 min. The proteins were precipitated, and the nucleic acid content of the samples was analyzed as before.

The influence of nucleoprotein concentration on the efficiency of nucleic acid removal by sulfitolysis is shown in Figure 4. The efficiency of nucleic acid removal was higher at low nucleoprotein concentrations. However, the efficiency decreased as the nucleoprotein concentration increased up to 2.5%. Above this concentration there was no further decrease in the efficiency of nucleic acid removal (Figure 4). It may be speculated that the apparent decrease in the efficiency may be due to either insufficient reduction and conversion of disulfide bonds to *S*-sulfonate derivatives or concentration-dependent increase in the nonspecific interaction between proteins and nucleic acids. However, analysis of these samples revealed that the number of disulfide bonds in all these protein samples was almost the same (Figure 4), indicating that the degree of sulfitolysis in all these samples was the same. This suggests that the decrease in the efficiency of nucleic acid removal may indeed be due to concentration-dependent nonspecific interaction between proteins and nucleic acids.

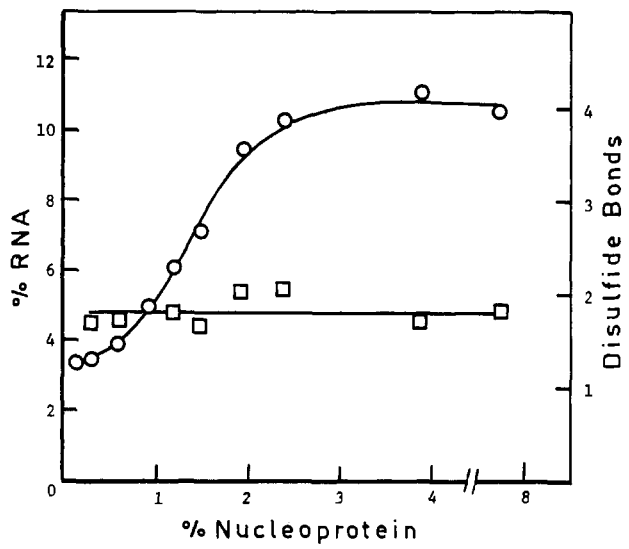

Figure 4. Effect of initial nucleoprotein concentration on the efficiency of removal of nucleic acids by sulfitolysis. The sulfite and tetrathionate concentrations used in the sulfitolysis procedure were 0.1 and 0.15 M, respectively. The initial nucleic acid content of the yeast protein sample was about 25%. ○ represents nucleic acid content. □ represents the number of disulfide bonds per 100 000 g of protein after sulfitolysis.

The results presented here indicate that the dissociation and removal of nucleic acids from yeast nucleoprotein complexes can be achieved by sulfitolysis. The basic mechanism involved in this approach is that reduction and conversion of disulfide bonds in nucleoproteins to *S*-sulfonate derivatives would induce conformational change and also would increase the electronegativity of the proteins. These in turn induce destabilization and dissociation of yeast nucleoprotein complexes. Since the dissociated nucleic acids have isoelectric pH around 1.5–2.0, they remain soluble during the isoelectric precipitation of the sulfonated yeast proteins at pH 4.2. This facilitates recovery of yeast proteins with low levels of nucleic acids.

The advantage of sulfitolysis over other chemical modification methods to decrease nucleic acid level (Shetty and Kinsella, 1979) is that those methods involve modification of lysyl residues in proteins, which decreases the bioavailability of lysine. In contrast, sulfitolysis involves only conversion of disulfides to *S*-sulfonate derivatives, and hence the bioavailability of the essential amino acids in sulfonated yeast proteins may not be impaired. However, this needs to be investigated. In view of recent controversies over the use of sulfites in foods (Nolan, 1983), it may be pointed out that the added sulfite and tetrathionate in the procedure described here can be removed completely during the isoelectric precipitation and washing or dialysis of the protein. Further, instead of tetrathionate any other suitable oxidizing agent can also be used. Since only very few disulfide groups (2/100 000 g of protein) are converted to *S*-sulfonate derivatives, and also desulfonation is achieved under acidic conditions (Bailey and Cole, 1959), the S-sulfonated proteins may be nutritionally safe. However, this needs further investigations.

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Detection of Added Whey Protein Concentrate in Nonfat Dry Milk by Amino Acid Analysis

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Existing procedures for the examination of nonfat dry milk (NDM) to detect added whey protein concentrate (WPC) are time consuming or require a great deal of sample handling. Amino acid analysis of NDM acid hydrolyzates with computer data handling is a method amenable to automation that will detect levels of >10% added WPC. Values in microgram percent for the amino acids aspartic acid, alanine, and proline are used as markers both in the screening procedure for simple acceptance or rejection of NDM samples and if desired the quantitative estimation of the extent of adulteration. The amino acid analysis results are not affected by heat treatment used in the NDM drying procedure, and the method is valid whether the WPC source is acid or sweet whey.

INTRODUCTION

Nonfat dry milk (also called skim milk powder) is widely used in the food industry and is a component of a wide spectrum of manufactured foods including baked goods, dairy products, and many other processed foodstuffs. Whey protein concentrates, produced in larger quantities in recent years as a result of changes in whey disposal practices, have a more limited commercial application and are available at a lower price. This makes it financially attractive to contravene federal regulations (Code of Federal Regulations, 1983) and adulterate nonfat dry milk (NDM) with whey protein concentrate (WPC). Since the nutritional parameters and functional properties of WPC differ from those of NDM (80% casein, 20% whey), it is important for regulatory agencies and food manufacturers to be able to detect such additions. Monitoring this adulteration is not a simple problem, since some WPC is formulated to be isoprotein and isolactose with NDM.

Neither the traditional Harland–Ashworth (1947) turbidimetric method for determination of undenatured whey proteins in heat-treated milk or the modification by Leighton (1962) can be successfully applied to quantitate WPC blended with NDM (Basch et al., 1985). Olieman and van den Bedem (1983) and van Hooydonk and Olieman (1982) developed a method for determining the amount of rennet (sweet) whey total solids in NDM by HPLC measurement of the glycomacropeptide (GMP) present. This procedure requires considerable "wet chemistry" (i.e., precipitation and filtration for each sample) and will not detect added acid whey powder produced by direct acidification. The gel electrophoretic procedure for whey quantitation reported by Basch et al. (1985) involves many manipulations, and there is a 3-day time factor for obtaining the results. Since the caseins that comprise the major protein fraction of NDM are quite low in cystine content, the finding of significant quantities of this amino acid is indicative of the presence of whey protein. Polarographic measurement of cystine (Mrowetz and Klostermayer, 1976) has therefore also been applied as a threshold index of added whey protein.

It is clear that there is a need for a routine screening procedure for detection and quantitation of added WPC in NDM, a procedure requiring a minimum of sample handling and amenable to automation. During extensive storage studies of NDM (Greenberg et al., 1977), we have observed that the amino acid profile with the exception of several sensitive amino acids is remarkably constant and is independent of the heat treatment used in the drying procedure. This report presents a method satisfying the aforementioned criteria and based on automatic amino acid analysis, which will permit the detection and/or quantitation of added WPC in NDM in a timely manner.

MATERIALS AND METHODS

Dry Milk and Whey. Skim milk powders (NDM) were sampled from lots stored by ASCS, USDA, in various locations throughout the U.S. The low- and high-heat NDM

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