Isolation of Proteins from Commercial Beet Sugar Preparations†

Robert L. Potter,*,‡ John D. Bacheller,§ Lee M. Chassy,§ and Richard L. Mansell§

Departments of Chemistry and Biology, University of South Florida, 4202 East Fowler Avenue, Tampa, Florida 33620-5250

Using a combination of ultrafiltration/dialysis and ion-exchange chromatography, we have isolated representative proteins from a variety of commercially available beet sugar preparations. Analysis by SDS-polyacrylamide gel electrophoresis gave Coomassie blue or silver staining bands ranging from 10 to 68 kDa with a prominent doublet at about 35 kDa. Treatment of the samples with protease resulted in the loss of all bands and a concomitant accumulation of staining at the dye front. More highly purified or refined sugar samples also contained similar proteins but in lesser amounts. Protein yields, as determined by the Coomassie blue dye binding method of Bradford, ranged from 1228 ng/g of sugar for some crystal samples to 40 ng/g of sugar for a highly refined liquor grade sample. Inverted sugar samples also contained many of the same proteins found in the crystalline samples.

Beet sugar is a relatively pure, multiuse food composed almost totally of sucrose. Its sweetness and relative cost make it a useful additive to a number of commercially available foodstuffs to enhance flavor and desirability. Instances have been described where individuals are apparently allergic to beet sugar taken orally (Randolf and Rollins 1950), or to sugar solutions administered parenterally (Richter et al., 1976), and yet not allergic to the major component, sucrose. Analysis of such an observation suggests that some component or components that copurify with sucrose during the crystallization procedure, or that are added during processing, might be the basis for the observed sensitivity. One report in the literature (Richter et al., 1976) using parenteral administration pointed to certain polysaccharide components as the immunogenic substance in some cases. Neill and coworkers (Neill et al., 1941) further demonstrated that some sugar samples contained material that reacted with antibodies directed toward specific cell surface antigens of certain bacteria (i.e., Type 2 pneumonococcus and *Leuconostoc mesenteroides*). This suggested that some of these bacteria (which are extremely heat resistant when encysted) or materials resulting from them may be present as a residue in sugar.

While using large amounts of sugar in our laboratory for macromolecule stabilization, we observed residues from concentrates of purified sugar that appeared to be proteinaceous in nature. Since beet sugar is extracted from plant materials which contain protein, we felt it was possible that some of the isolated protein could be of plant origin. Alternatively, this material could have arisen from bacteria associated with beets before or during sugar processing. To better understand this phenomenon, we therefore undertook an investigation into the residual protein found in commercial beet sugar and report the findings herein.

† This work was supported by grants from the Florida Department of Citrus and the Processed Apples Institute.

* To whom correspondence should be addressed.

‡ Department of Chemistry.

§ Department of Biology.

MATERIALS AND METHODS

Sugar Samples and Reagents. Crystalline sugar was purchased from Spreckels Sugar Co., both the Woodland, CA, and Pleasanton, CA, facilities, as well as from Amalgamated Sugar Co., Nampa, ID, and Twin Falls, ID. European crystal came from Weiszucker EG Qualitaet II Fuellgewicht Zuckerfabrik Juelich AG, West Germany, and Fa. Steirobst GesmbH, Austria. Inverted sugar samples came from Spreckels Sugar Co., Woodland, CA; Sudzucker AG, West Germany, and Amalgamated Sugar Co., Oak Grove, IL. All other chemicals used were of reagent grade with sodium azide, thiourea, ammonium bicarbonate, and sodium phosphate purchased from Fisher Scientific, Fairlawn, NJ. Mercaptoethanol was obtained from Sigma Chemical Co., St. Louis, MO.

Protein Isolation. Beet sugar proteins were isolated as follows. Beet sugar, from various commercial sources, was prepared as a 15° Brix solution in medium A (0.1% sodium azide, 5 mM thiourea, and 2 mM β-mercaptoethanol). Following vacuum filtration through a 0.45-μm Supor 450 membrane filter (Gelman Sciences), the sugar solution was dialyzed by countercurrent dialysis using three Spectra/Por RC hollow fiber bundles (MWCO 6000) linked in series. The dialyzing medium was identical with medium A.

Following dialysis, 4 L of the approximately 2° Brix sugar solutions was loaded at 4 °C onto each of five small anion-exchange columns (1 mL bed volume PEI silica SPE-10, J. T. Baker Chemical Co.). Material was eluted at room temperature with 2.5 column volumes of 550 mM ammonium bicarbonate, pH 7.8, and the resulting eluate from five columns was combined and concentrated as described below. The samples at this stage were characterized by a light straw to dark golden yellow color.

Protein Concentration. The column eluant was reduced under pressurized nitrogen gas (30 psi) to 2 mL volume by using a Novacell NMWL 10000 stirred pressure cell (Filtron Technology Corp.). The retentate was further concentrated and desalted by a series of centrifugations at 5000 rpm in a Centricon 10000 MWCO microconcentrator (Amicon Division, W. R. Grace Co.). Following each spin, the retentate was diluted to double volume by addition of a 0.1% sodium azide and 2 mM mercaptoethanol solution and reconcentrated. This process was repeated six times.

Protein Determination. Protein concentration was determined by the Coomassie blue dye binding assay (Bradford, 1976) using the Bio-Rad protein assay kit 1 (Bio-Rad Laboratories). Bovine plasma γ-globulin (Bio-Rad) was used as the protein standard.

Protease Treatment. Samples were treated with papaya protease (Sigma) 50:1 (w/w) at either 4 °C or room tempera-

0021-8561/90/1438-1498$02.50/0

ture for 19 min. The reaction was terminated by the addition of SDS followed immediately by boiling for 2 min.

Polyacrylamide Gel Electrophoresis. Beet sugar proteins were fixed (2:1) in SDS denaturing solution (2.5% SDS, 25% sucrose, 0.25 mg/mL pyronin Y, 2.5 mM EDTA, 215 mM β-mercaptoethanol), and the resulting protein samples were separated on a 1 mm 10% acrylamide gel prepared as described by Laemmli (1970). Gels were run at room temperature with a constant current of 10 mA.

Gels were fixed in 10% isopropyl alcohol and 5% acetic acid for 10 min and stained in the same solution with 2.5% Coomassie blue R-250. Destaining took place in 10% isopropyl alcohol and 5% acetic acid plus 3% glycerol. Gels were placed onto Whatman 3-mm paper and dried under vacuum.

Protein molecular weights were estimated by the method of Weber and Osborn (1969) using molecular weight standards from Bio-Rad. The marker proteins included phosphorylase *b* (97 400), bovine serum albumin (66 200), ovalbumin (42 699), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400).

Amino Acid Analysis. Samples were hydrolyzed in duplicate for 24 h at 110 °C in 6 N HCl, and then the acid was removed by rotary evaporation. Amino acids were quantitated after derivatization with phenyl isothiocyanate and separated by reverse-phase high-performance liquid chromatography (Heinrikson and Meredith, 1984). The protein angiongenin was used as the standard for quantitation.

RESULTS

A protein-containing fraction was isolated from a variety of commercially available beet sugar preparations (more than 12). The isolation procedure included a flow-through dialysis step to lower the sucrose concentration followed by chromatography on a weak anion exchanger. The final preparation was obtained by batch elution from the ion-exchange column followed by concentration and washing of the eluted sample via ultrafiltration. The resulting, slightly yellow sample was analyzed by polyacrylamide gel electrophoresis and Coomassie blue dye staining. A typical profile can be seen in Figure 1. More than 20 Coomassie blue staining bands can be seen with prominent bands at 61, 45, 35, 30, 27, and 20 kDa.

The proteinaceous characteristics of this staining material can be inferred from the experiments shown in Figure 2 and from Table I. When the protein samples were exposed to a mixture of papaya protease prior to electrophoresis, the stained bands were no longer seen but were replaced by a heavy, diffuse staining around the dye front (Figure 2).

Amino acid analysis of the isolated material (Table I) also indicated the presence of macromolecular polymers of amino acids linked by acid labile bonds. Greater than 50% of the mass of the material could be recovered as standard protein amino acids (36 μg of amino acids from 66 μg of starting material). Several other unidentified peaks were also evident in the analysis profile and may account for some of the remaining mass (data not shown). The amino acid profile would seem to indicate proteins rich in alanine, serine, and glycine and, interestingly, relatively rich in proline.

The similarity of proteins isolated from different sugar sources can be seen in Figure 3, which shows an electrophoretic comparison of material isolated from several different sugar products. Samples from each source have been isolated a minimum of three times with results identical with those illustrated. Lanes B–D are invert sugar samples from Spreckels Sugar Co. in California (invert sugar means some proportion of the sucrose has been hydrolyzed to fructose and glucose), lanes G–I are three different crystal sugar samples, including one from Austria (lane G). Note that all of these samples show overlapping

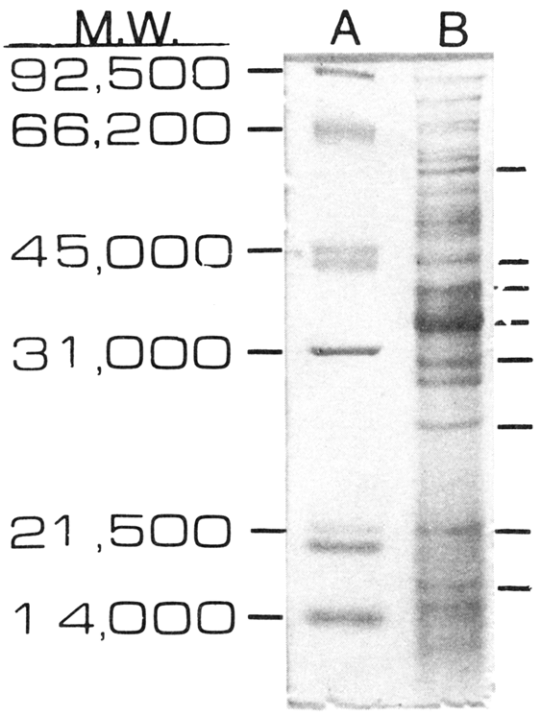

Figure 1. Electrophoretic profile of crystal beet sugar protein extract. Coomassie blue stained SDS–10% polyacrylamide gel. (Lane A) Low molecular weight marker with weights on left. (Lane B) 9 μg of protein extract from crystal beet sugar (Amalgamated Sugar Co.). Major protein bands in extract are indicated by hatch marks on right. Estimated molecular weights (top to bottom) are 61 000 (doublet), 45 000, 39 000, 35 000, 30 000 (doublet), 27 000, 21 000, and 16 000.

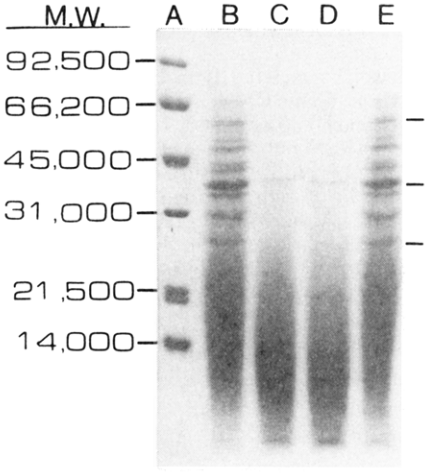

Figure 2. Electrophoretic analysis of protease-treated vs untreated crystal beet sugar protein extract. Coomassie blue stained SDS–10% polyacrylamide gel. Crystal beet sugar (Amalgamated Sugar Co.) protein extract was treated with a 10:1 ratio (w/w) of protease from papaya (Sigma Chemical Co.) and incubated for 19 min at the indicated temperature; the reaction was stopped by addition of PSM. (Lane A) Low molecular weight markers with weights on left. (Lane B) 13 μg of untreated protein extract incubated at room temperature. (Lane C) 13 μg of protease-treated protein extract incubated at room temperature. (Lane D) 13 μg of protease-treated protein extract incubated at 4 °C. (Lane E) 13 μg of untreated protein extract incubated at 4 °C. Major protein bands in extract are indicated by hatch marks on right. Estimated molecular weights (top to bottom) are 61 000 (doublet), 35 000, and 27 000.

patterns of protein which in some cases look almost identical. The major protein bands evident in Figure 1 can also be clearly seen in these samples. The few differences evident may simply reflect differences in how

Table I. Amino Acid Analysis of Protein Extract from Crystal Beet Sugar (Amalgamated Sugar Co.)

amino acid	mol % sample 1	mol % sample 2
Asp	7.9	8.8
Glu	6.4	6.6
Ser	10.4	11.3
Gly	10.2	13.0
His	1.3	1.2
Arg	1.8	1.6
Thr	6.6	6.4
Ala	14.0	14.1
Pro	9.6	9.0
Tyr	3.0	2.8
Val	7.3	6.4
Met	2.3	2.2
Ile	4.3	4.2
Leu	7.8	6.2
Phe	3.6	3.2
Lys	3.5	3.2

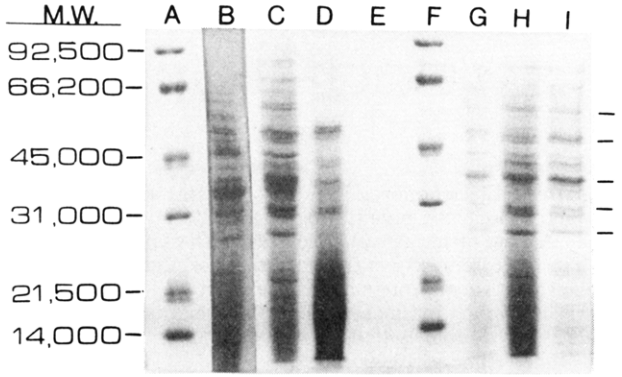

Figure 3. Electrophoretic profiles of protein extracts from various crystal and invert beet sugars. Coomassie blue stained SDS–10% polyacrylamide gel. (Lanes A and F) Low molecular weight markers with weights on left. (Lane B) 13 μg of 50% invert (Spreckels Sugar Co.). (Lane C) 24 μg of 78% Invert (Amalgamated Sugar Co.). (Lane D) 30 μg of 90% Invert (Spreckels Sugar Co.). (Lane E) Blank (PSM only). (Lane G) 15 μg of Crystal (Austria). (Lane H) 37 μg of Medium Grade Crystal (Amalgamated Sugar Co.). (Lane I) 7 μg of highly refined liquor grade Crystal (Spreckels Sugar Co.). Major protein bands in protein extracts are indicated by hatch marks on right. Estimated molecular weights (top to bottom) are 61 000 (doublet), 45 000, 35 000, 30 000 (doublet), and 27 000.

they are produced and are most clearly seen in Figure 4. While most of the protein bands (Figure 4, lanes B–D) appear to be similar to those isolated from crystal sugar, the predominant band in these inverted samples appears to be one at 30 kDa along with lesser quantities of the usually dominant 35-kDa protein seen in the pattern from crystal (Figures 4 or 3). Thus, while some inverts do differ, they still show overlapping protein patterns, and all sugar samples possess a protein band of about 30 kDa among others.

As with the protein profiles, yields of protein varied with the area of origin and also with the type and purity of the specific commercial samples used. Typical yields can be seen in Table II and are the average of at least three isolations. Crystalline samples contained the highest amounts of protein (1228 ng/g), whereas more highly purified or treated sugars such as liquor grade or invert samples contained the least (50–350 ng/g).

DISCUSSION

We have presented evidence for the existence of discrete proteinaceous substances that can be purified from commercial beet sugar preparations. These proteins have

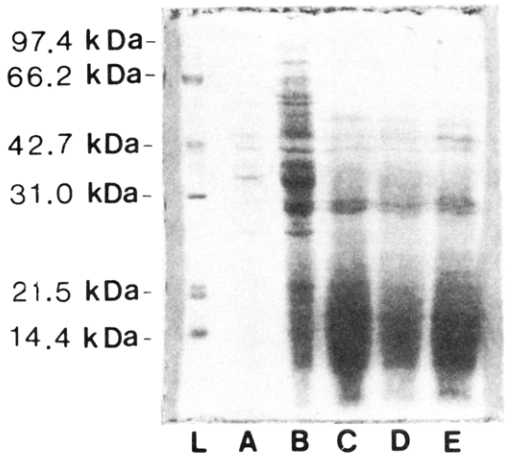

Figure 4. Electrophoretic profiles of protein extracts from various crystal and invert beet sugars. Coomassie blue stained SDS–10% polyacrylamide gel. (Lane L) Low molecular weight markers with weights on left. (Lane A) 7 μg of highly refined liquor grade Crystal (Spreckels Sugar Co.). (Lane B) 9 μg of medium grade Crystal (Amalgamated Sugar Co.). (Lane C) 69 μg of 50% invert (Amalgamated Sugar Co.). (Lane D) 29 μg of 78% invert (Amalgamated Sugar Co.). (Lane E) 60 μg of 90% invert (Amalgamated Sugar Co.).

Table II. Protein Yields from Beet Sugars of Various Types and Sources

type	yield/g sugar[a]	source
crystal	1228 ± 495 ng	White Satin Brand 12N110100H Amalgamated Sugar Co., Nampa, ID
crystal	760 ± 248 ng	White Satin Brand 12N07G704H Amalgamated Sugar Co., Nampa, ID
crystal	685 ± 149 ng	Spreckels MXR, Spreckels Sugar Co., Pleasantville, CA
crystal, highly ref	54 ± 10 ng	Amstar Brand, Spreckels Sugar Co., Pleasantville, CA
crystal, European	105 ng[b]	Weiss Zuckfer, EG-Qualitat II, Fullgewicht, Zuckerfabrik Julich AG, West Germany
50% invert	566 ± 100 ng	Amalgamated Sugar Co., Oak Grove, IL
50% invert	163 ± 112 ng	Spreckels Sugar Co., Woodland, CA
67% invert, European	43 ● 2 ng	Sudzuker AG, West Germany
78% invert	477 ± 170 ng	Amalgamated Sugar Co., Oak Grove, IL
90% invert	158 ng[b]	Amalgamated Sugar Co., Oak Grove, IL
90% invert	285 ● 56 ng	Spreckels Sugar Co., Woodland, CA

[a] Protein concentrations determined by Bradford protein assay. [b] Single sample.

been isolated from a number of domestic crystalline beet sugar samples as well as from European beet sugar and domestic inverted samples.

The protein character of the isolated material was established in two ways. The material was susceptibile to protease treatment, as shown by the loss of discrete Coomassie blue staining bands on polyacrylamide gels. The material also yielded amino acids following vigorous acid hydrolysis. The amount of free amino acids liberated was consistent with protein from these building blocks having formed the major portion of the isolated material. During the amino acid analysis several unidentified peaks were also seen and may represent amino acids that were chemically altered during the rather harsh beet sugar extraction and purification procedure (Ebell and Strong, 1982; McGinnis, 1982). It is quite possible that some or

all of these may be so-called Maillard compounds generated from the reaction of amino acids with sugars shown to be present in heated solutions containing amino acids and sugars (Stegnick et al., 1981). These peaks were not calculated in the amino acid recovery and could, if they are modified amino acids, add to the 54% recovery. Furthermore, the initial quantitation of protein is based on the Coomassie blue dye binding method of Bradford, which is known to vary for different proteins (Davis, 1988). The actual amount of protein hydrolyzed could have been overestimated if some portion of the isolated material is not protein but interferes with the dye binding assay. Certain flavonoid substances, for instance, are known to do this, thereby giving elevated protein values (Compton and Jones, 1988). In terms of the amino acid makeup, since the analysis is derived from a mixture of proteins, the interpretations are limited; however, the relatively high amount of serine and threonine (16%) is consistent with the type of amino acids that could interact with the hydrogen-bonding potential of sucrose.

The amounts of protein found in different crystal preparations were small, but consistent, from batch to batch ranging from 1228 to 54 ng/g of sugar depending on the producer. Highly refined liquor grade sucrose appears to have less total protein which is probably due to the increased number of purification steps required to obtain this product. The refined sugar still contains the same major protein bands as seen in the other samples (61, 45, 35, 30, and 27 kDa). Thus, while the total amount of protein per gram of sample seems to change from producer to producer and with the amount of processing, the qualitative pattern remains remarkably similar.

It is interesting to note that some of the invert sugar samples show a decreased amount of the protein band at 35 kDa and a relative increase in the protein band at 30 kDa. Since invert sugar (sugar that has had some sucrose hydrolyzed to fructose and glucose) is produced by more than one method (Gaddie, 1982), these differences may simply reflect the different sugar-processing procedures. Most invert production involves treatment of sucrose solutions with mild HCl for varying lengths of time and at various temperatures (Gaddie, 1982). Thus, the differences in the profiles of the proteins isolated from the sugars processed by the various methods might be explained by selective hydrolysis or differential precipitation of certain proteins that change as a result of the procedure used for inversion. For instance, selective hydrolysis at certain more susceptible amide bonds (i.e., aspartic acid–proline; Laurson, 1977; Allen, 1981) could lead to loss of one or more larger proteins with concomitant increases in one or more smaller ones. This possibility is enhanced by the large percentage of proline and potential aspartic acid residues found in these proteins. Alternatively, the changed protein profile could be due to preferential loss of the 35-kDa protein during the acid treatment via denaturation and precipitation. In any case, several proteins of similar molecular weight can be isolated from both the inverted and regular sugar samples.

Since protein was found in all sugar samples tested, it was important to demonstrate that the protein material was a consistent component of the sugar samples and not endogenously produced during our isolation procedure. Several lines of evidence support this conclusion. First, all samples were maintained in 0.1% sodium azide, which is known to be an effective inhibitor of aerobic bacterial growth. Second, an equivalent portion of buffer, in which the sugar is normally dissolved, failed to show any Coomassie or silver staining bands on SDS–PAGE when processed through our procedure. Third, the same protein bands can be obtained by passing the 15° Brix solution directly through ion-exchange columns without a dialysis step (data not shown). While the yields of the proteins are reduced, possibly due to the elevated sucrose interfering with binding, the fact that they can still be found suggests they are not generated in situ. The nonbinding protein can also be recovered if this fraction is dialyzed and concentrated and the resulting protein profile appears similar to that seen for the initial sample (data not shown). Finally, the fact that some acid inverted sugar extracts show a different quantitative pattern of proteins on SDS–PAGE from that shown by crystalline extracts strongly supports the idea that these proteins are specifically associated with the commercial sugar preparations and are not being generated during the isolation procedure.

While we have established the existence of small amounts of specific proteins in all of the commercial beet sugar preparations tested, the origin of these macromolecules remains unknown. Since these proteins are found in sugars from such diverse areas as California, Utah, Germany, and Austria, the most likely candidate source would seem to be the beet plant material, although contamination of beets or beet sugar with specific microorganisms (Halden and Bolinder, 1982) cannot be ruled out. We are presently investigating these possibilities using antibodies raised against the isolated beet sugar proteins.

ABBREVIATIONS USED

Invert sugar, sucrose that has been partially hydrolyzed to fructose and glucose; PAGE, polyacrylamide gel electrophoresis; PEI, poly(ethylene imine); SDS, sodium dodecyl sulfate.

ACKNOWLEDGMENT

We express our appreciation to Dr. Peter Neame, Chief of Protein Structure Section, Shriners Hospital, Tampa, for the amino acid analysis and to Donna Hux and Dick Courtney for their expert technical assistance. We thank Jo Aaron for her efficient and careful preparation of the manuscript.

LITERATURE CITED

Allen, G. Sequencing of Proteins and Peptides. *Lab Tech. Biochem. Mol. Biol.* **1981**, *9*, 50.

Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Compton, S. J.; Jones, C. G. Mechanism of Dye Response and Interference in the Bradford Protein Assay. *Anal. Biochem.* **1985**, *151*, 369–374.

Davis, E. M. Protein Assays: A review of common techniques. *Biotechnol. Lab.* **1988**, *6* (5), 28–37.

Duke, W. W. *Asthma, Hay Fever, Urticaria and Allied Manifestations of Allergy*; Mosby: St. Louis, MO, 1926.

Ebell, H. O.; Story, M. Diffusion. In *Beet Sugar Technology*; McGinnis, R. A., Ed.; 1982; Chapter 6, pp 119–132.

Gaddie, R. S. Liquid Sugars. In *Beet Sugar Technology*; McGinnis, R. A. Ed.; 1982; Chapter 15, pp 537–544.

Goding, J. W. *Monoclonal Antibodies: Principles and Practice*; Academic Press: Orlando, FL, 1983; pp 114–115.

Halden, H. E., revised by Bolinder, M. Microbiology and Plant Sanitation. In *Beet Sugar Technology*; McGinnis, R. A., Ed.; 1982; Chapter 23, pp 723–740.

Heinrickson, R. L.; Meredith, S. C. Amino Acid Analysis by Reverse-Phase High-Performance Liquid Chromatography: Precolumn Derivatization with Phenylisothiocyanate. *Anal. Biochem.* **1984**, *136*, 65–74.

Laemmli, U. K. Cleavage of the structural proteins of the bacteriophage T-4. *Nature (London)* **1970**, *227*, 680–685.

Laursen, R. A. Automatic solid phase Edman degradation. *Methods Enzymol.* **1977**, *25*, 344.

McGinnis, R. A. Juice Purification. In *Beet Sugar Technology*; McGinnis, R. A., Ed.; 1982; Chapter 7, pp 155–184.

Neill, J. M.; Sugg, J. Y.; Hehre, E. J.; Jaffe, E. Serological studies on sugar. Reactions of antiserums of Type II pneumonococcus and of Leuconostoc mesenteroides with cane and beet sugars and with cane juice. *Am. J. Hyg.* **1941**, *34*, 65–78.

Randolph, T. G.; Rollins, J. P. Beet sensitivity: Allergic reactions from the ingestion of beet sugar (sucrose) and monosodium glutamate of beet origin. *J. Lab. Clin. Med.* **1950**, *36*, 407.

Richter, A. W.; Granath, K.; Ostling, G. Anaphlactoid reactions in connection with infusion of invert sugar solutions are due to macromolecular contaminants. *Int. Arch. AllergyAppl. Immunol.* **1976**, *50*, 606.

Stegink, L. D.; Freeman, J. B.; Don Barsten, L.; Filer, L. J., Jr. Maillard Reaction Products in Parenteral Nutrition. *Prog. Food Sci.* **1981**, *5*, 265–278.

Weber, K.; Osborn, M. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **1969**, *244*, 4406.

Received for review June 22, 1989. Accepted February 1, 1990.

Registry No. Sucrose, 57-50-1; invert sugar, 8013-17-0.