the carbon peaks. The separation needs, however, not to be complete, because any modern chromatographic integrator computes a complete separation also when the base line is not reached between the peaks.

Calculation of the Results. The dry weight of the sample is $C \times F_c \times 100/D$, where C is the area of the carbon peak, F_c is the calibration factor defined by $C \times F_c$ = mg of carbon, and D is the percentage of carbon in dry grain. D is read from Table I.

The sample's content of nitrogen can, therefore, be calculated with the equation

$$\% \text{ N} = N \times F_n \times D/(C \times F_c) \quad (5)$$

in which N is the area of the nitrogen peak and F_n the calibration factor for nitrogen defined by $N \times F_n$ = mg of N.

The sample's content of sulfur and hydrogen is calculated in the same manner with S and F_s and H and F_h instead of N and F_n.

For the calculation of the moisture content we have to substract the hydrogen content of the dry grain—E, read from Table I—from the total hydrogen content of the sample to obtain the water hydrogen, which we convert to water with the factor 9. Hence, we obtain

$$\% \text{ moisture in the } \textit{dry} \text{ grain} = 9[H \times F_h \times D/(C \times F_c) - E] \quad (6)$$

The percentage of moisture in the fresh grain is then given by

$$\% \text{ moisture} = \frac{100 \times 9[H \times F_h \times D/(C \times F_c) - E]}{100 + 9[H \times F_h \times D/(C \times F_c) - E]} \quad (7)$$

In eq 5, 6, and 7 we can set $F_n/F_c = A$, $F_h/F_c = B$, and $F_s/F_c = C$.

Quotients A, B, and C are obtained from the calibration analyses:

$$A = \% \text{ N} \times \text{peak area of C}/(\% \text{ C} \times \text{peak area of N})$$

$$B = \% \text{ H} \times \text{peak area of C}/(\% \text{ C} \times \text{peak area of H})$$

$$C = \% \text{ S} \times \text{peak area of C}/(\% \text{ C} \times \text{peak area of S})$$

When we introduce A and B into eq 5 and 7, we obtain the calculation formulas 1 and 2, reported above in the description of the analytical method.

Equation 3 is based upon the assumption that the presence of moisture in the sample causes a corresponding deviation of its carbon content from that of Table I.

Equation 4 is a mere correction of the sample's nitrogen content for its moisture content.

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Registry No. Carbon, 7440-44-0; nitrogen, 7727-37-9; hydrogen, 1333-74-0; water, 7732-18-5.

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Determination of Protein Hydrophobicity Using a Sodium Dodecyl Sulfate Binding Method

Akio Kato,* Tomohiko Matsuda, Naotoshi Matsudomi, and Kunihiko Kobayashi

An attempt to determine the protein hydrophobicity was made by using the sodium dodecyl sulfate (SDS) binding method. The SDS-binding capacity of proteins in the presence of a low concentration of SDS was proportional to the surface hydrophobicity determined by the fluorescence probe method. The electrostatic effect on the SDS-binding capacity was not observed. As protein denaturation proceeded, the SDS-binding capacity of proteins changed correspondingly to changes in the protein hydrophobicity. A good correlation was observed between the surface hydrophobicity and SDS-binding capacity of 42 native and denatured proteins. The SDS-binding method was applied to determine the surface hydrophobicity of insoluble denatured ovalbumins. It was suggested that this method was suitable for the determination of the surface hydrophobicity of insoluble proteins.

Attempts to determine the surface hydrophobicity of proteins have been made by some investigators (Shanbhag and Axelsson, 1975; Keshavarz and Nakai, 1979; Kato and Naki, 1980). Shanbhag and Axelsson (1975) established the hydrophobic partition method to determine the surface hydrophobicity of proteins. Keshavarz and Nakai (1979) applied hydrophobic chromatography to assess the surface hydrophobicity. Kato and Nakai (1980) reported the

Department of Agricultural Chemistry, Yamaguchi University, Yamaguchi 753, Japan.

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method to measure the surface hydrophobicity of proteins using *cis*-parinaric acid as a fluorescence probe. These methods can be used for the soluble protein systems but not for the insoluble protein systems. It is desirable to develop a method that can be applied to assess the surface hydrophobicity of insoluble proteins, because of the requirement for the determination of hydrophobicity of insoluble protein systems, such as gels and aggregates. For this purpose, the method using the interaction between protein and sodium dodecyl sulfate (SDS) seems to be promising. SDS has been believed to be a drastic denaturant for proteins. However, many enzymes are active in the presence of considerably high concentrations of SDS (Nelson, 1971). In some cases, the helix conformation of proteins increases in the presence of SDS (Mattice et al., 1976) and in other cases, detergents protect the proteins from denaturation (Stewart et al., 1974). Imoto et al. (1979) reported that SDS formed a specific complex with lysozyme without causing a gross conformational changes in the molecule and that hydrophobic regions and positive charges of lysozyme were required for the interaction.

This paper describes the relationship between the SDS-binding capacity and surface hydrophobicity of proteins. In addition, the role of SDS as a hydrophobic probe for proteins is discussed.

MATERIALS AND METHODS

Ovalbumin was prepared from fresh egg white by the sodium sulfate procedure (Kekwick and Cannan, 1936) and recrystallized 5 times. Lysozyme was prepared from fresh egg white by the direct crystallization method (Alderton and Fevold, 1946) and recrystallized 5 times. Ovomucoid was prepared from fresh egg white by the method of Waheed and Salahuddin (1975). Ovotransferrin was prepared from fresh egg white by the method of Azari and Baugh (1967). κ-Casein was prepared from fresh milk by the method of Zittle and Custer (1963). Soybean 7S and 11S globulin (7S and 11S globulin) was prepared by the method of Thanh et al. (1975). Bovine serum albumin, trypsin (pancreatic type II), catalase (from bovine liver), and lipase (type I from wheat germ) were purchased from Sigma Chemical Co. (St. Louis, MO). β-Lactoglobulin (bovine) was purchased from ICN Pharmaceuticals Inc. (Cleveland, OH). Ribonuclease (bovine pancreas) was purchased from P-L Biochemicals (Milwaukee, WI). γ-Globulin (bovine) was purchased from Miles Laboratories (United Kingdom).

The heat denaturation of proteins was carried out as follows: 10 mL of a 0.1% protein solution in 0.02 M phosphate buffer, pH 6.0, was heated in an incubator at an increasing rate of 1 °C/min from 20 to 80 °C. Heat-denatured protein solution was immediately cooled to 20 °C after the rise to a given temperature.

The SDS-binding capacity was determined as follows: SDS was added in 10 mL of a 0.1% protein solution and adjusted to 0.07 mM. After being allowed to stand 30 min, SDS–protein mixtures were dialyzed against 25 volumes of 0.02 M phosphate buffer, pH 6.0, for 24 h. The SDS contents of inner dialyzates were determined by Epton's method (1948). Ten milliliters of $CHCl_3$ was added to 0.5 mL of inner dialyzates and mixed in a test tube. Then 2.5 mL of a 0.0024% methylene blue solution was added to the $CHCl_3$ layer. After being mixed in the test tube, the mixture was centrifuged at 2500 rpm. The absorbance of the SDS–methylene blue mixture in the lower layer was measured at 655 nm. Thus, SDS-binding capacity was determined and was represented as micrograms of SDS bound to 500 μg of protein.

The measurement of surface hydrophobicity of proteins was carried out using *cis*-parinaric acid as a fluorescence probe (Kato and Nakai, 1980). Ethanolic solutions of *cis*-parinaric acid, 3.6×10^{-3} M, were purged with nitrogen and equimolar butylated hydroxytoluene was added as an antioxidant. Ten microliters of the *cis*-parinaric acid solution was added to 2 mL of the protein solution in 0.02 M phosphate buffer, pH 6.0. The parinaric acid–protein conjugates were excited at 325 nm and the relative fluorescence intensity was measured at 420 nm in an Aminco-Bowman spectrophotofluorometer, J4-8962. The relative fluorescence intensity reading was adjusted to 1.0 when 10 μL of the *cis*-parinaric acid solution was added to 2 mL of 0.02 M phosphate buffer, pH 6.0, in the absence of protein. The initial slope (S_0), fluorescence intensity/percent protein, was calculated from the fluorescence intensity vs. protein concentration plot.

In measurement of the surface hydrophobicity and SDS-binding capacity of insoluble ovalbumin, the samples were thoroughly homogenized with a homogenizer (Ultra Turrax, Hansen & Co.) for 1 min at 12000 rpm and then SDS or *cis*-parinaric acid was added to the homogenized samples.

Table I. Relationship between Surface Hydrophobicity and SDS-Binding Capacity of Proteins

protein	hydrophobicity (S_0)	binding of SDS, μg
ovalbumin	15	1.4
lysozyme	25	1.8
ribonuclease	30	1.7
ovomucoid	59	1.7
γ-globulin	83	1.9
trypsin	94	1.3
11S globulin	96	2.0
ovotransferrin	114	1.9
catalase	210	2.3
lipase	240	2.1
7S globulin	247	2.6
κ-casein	358	2.9
β-lactoglobulin	2322	6.0
bovine serum albumin	2729	9.7

RESULTS AND DISCUSSION

A very low concentration (0.07 mM) of SDS was used to determine the SDS-binding capacity of proteins. This concentration is $^1/_{40}$ of critical micelle concentration (2.8 mM) (Steinhardt et al., 1977) and is the saturating one for bovine serum albumin, which has the highest SDS-binding capacity. It is not probable that proteins denature in such a low concentration of SDS. Therefore, only SDS attached to the molecular surface of proteins may be determined in this experiment. Table I shows the relationship between the SDS-binding capacity and surface hydrophobicity of various proteins. The SDS-binding capacity of proteins increases with the surface hydrophobicity. A good correlation was observed between the SDS-binding capacity and surface hydrophobicity of proteins and the correlation coefficient was 0.97 ($p < 0.01$). However, a poor correlation was observed at low SDS-binding values. Probably, this method only works well for proteins that bind large amounts of SDS.

Figure 1 shows the effect of pH on SDS-binding capacity and surface hydrophobicity of 11S globulin, ovotransferrin, ovalbumin, lysozyme, bovine serum albumin, and β-lactoglobulin. The pH dependence of SDS-binding should be the same for all proteins only if the pH dependence of the protein surface charge is the same for all the proteins. This seems unlikely for the proteins listed in Figure 1. The SDS-binding capacity of ovalbumin decreased with a pH increase, while that of lysozyme, bovine serum albumin,

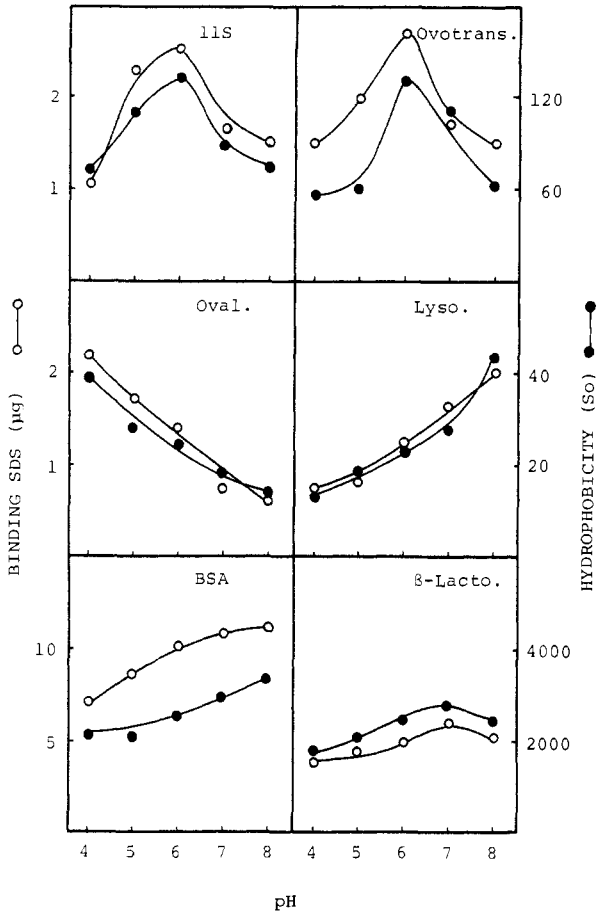

Figure 1. Effect of pH on surface hydrophobicity and SDS-binding capacity of proteins.

and β-lactoglobulin increased with a pH increase. In addition, the SDS-binding capacity of 11S globulin and ovotransferrin was maximum around at pH 6.0. Thus, the common dependence of pH on the SDS-binding capacity was not observed for six proteins. On the other hand, the SDS-binding capacity of proteins changed correspondingly to the surface hydrophobicity. Figure 2 shows the effect of ionic strength on SDS-binding capacity and surface hydrophobicity of 11S globulin, ovotransferrin, ovalbumin, lysozyme, bovine serum albumin, and β-lactoglobulin. The SDS-binding capacity of 11S globulin, ovalbumin, and lysozyme increased with ionic strength, while that of ovotransferrin, bovine serum albumin, and β-lactoglobulin showed maximal values about at 0.2. As described above, if the interaction of proteins with SDS is due to the electrostatic bond, the SDS-binding capacity should decrease with an increase in ionic strength. However, this tendency was not observed in Figure 2. Interestingly, the SDS-binding capacity changed correspondingly to the surface hydrophobicity of proteins, as shown in Figure 1. Thus, the importance of hydrophobic interaction was suggested in the SDS-binding capacity of proteins.

Changes in the SDS-binding capacity of proteins were investigated during heat denaturation. Figure 3 shows changes in the surface hydrophobicity and SDS-binding capacity of bovine serum albumin, ovotransferrin, 11S globulin, and ovalbumin during heat denaturation. The SDS-binding capacity of ovotransferrin, 11S globulin, and ovalbumin greatly increased as heat denaturation proceeded, while that of bovine serum albumin decreased with heat denaturation, corresponding to the surface hydrophobicity. Good coreelations were observed between the surface hydrophobicity and SDS-binding capacity of de-

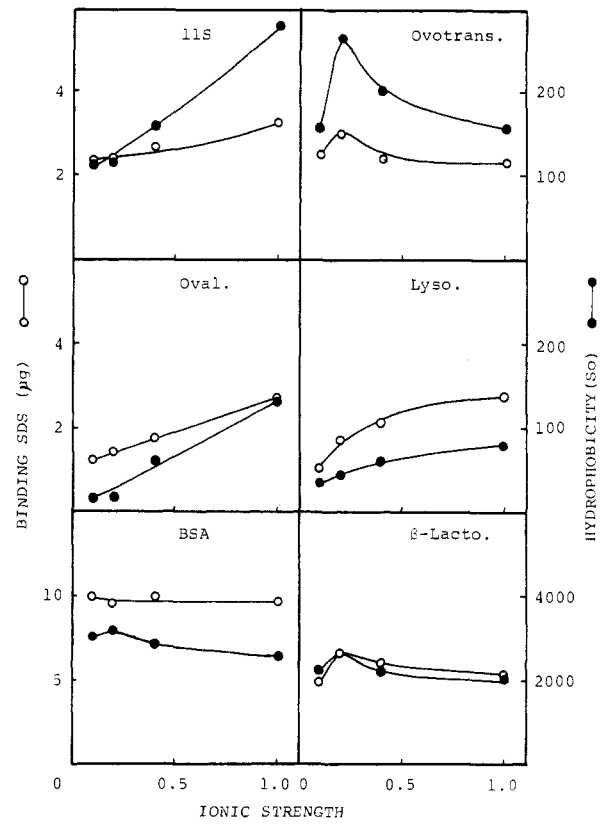

Figure 2. Effect of ionic strength on surface hydrophobicity and SDS-binding capacity of proteins.

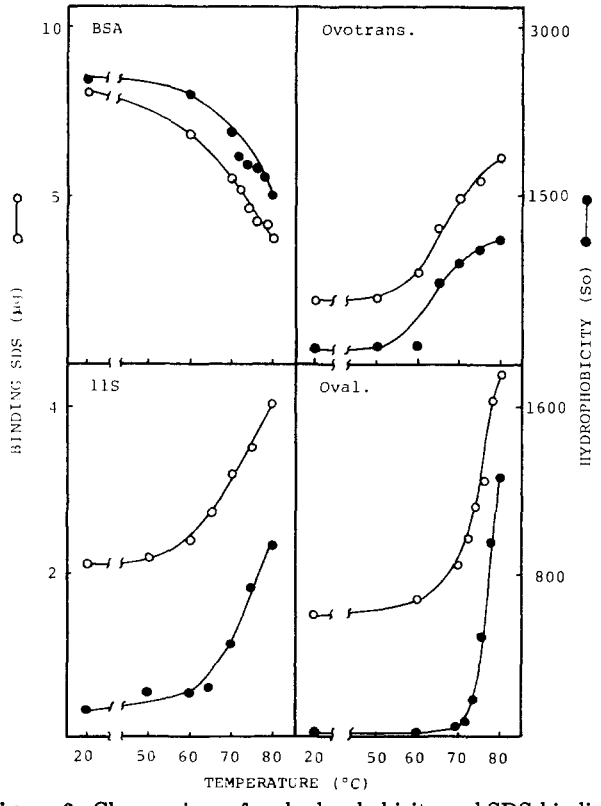

Figure 3. Changes in surface hydrophobicity and SDS-binding capacity of proteins during heat denaturation.

natured albumins, ovotransferrins, 11S globulins, and ovalbumins, as shown in Figure 4. The correlation coefficients are 0.97, 0.98, 0.97, and 0.95 ($p < 0.01$) for denatured albumins, ovotransferrins, 11S globulins, and ovalbumins, respectively. The dependence of SDS-binding

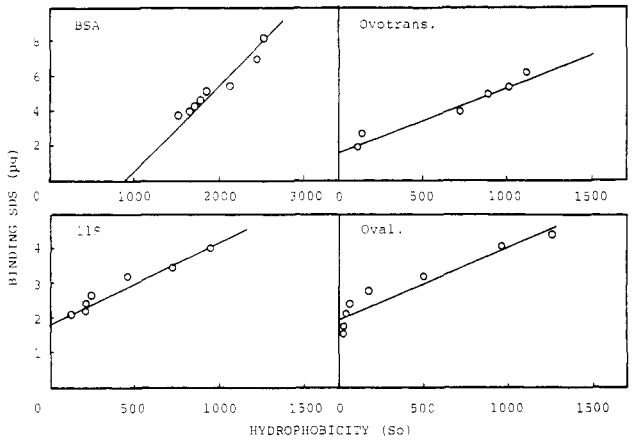

Figure 4. Correlation between surface hydrophobicity and SDS-binding capacity of heat-denatured proteins.

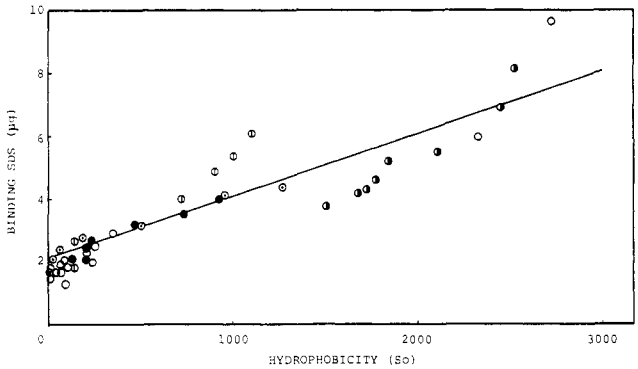

Figure 5. Correlation between surface hydrophobicity and SDS-binding capacity of various proteins. (○) Native proteins in Table I; (⊙) denatured ovalbumins; (◑) denatured bovine serum albumins; (◐) denatured ovotransferrins; (●) denatured 11S globulins.

capacity on surface hydrophobicity is suggestive of the importance of hydrophobic interaction in SDS-protein binding.

Figure 5 shows the relationship between the surface hydrophobicity and SDS-binding capacity of various proteins. Although native protein and denatured protein systems were combined, a good correlation was observed between the surface hydrophobicity and SDS-binding capacity. The correlation coefficient is 0.92 ($p < 0.01$) and the regression equation is $Y = (2.12 \times 10^{-3})X + 1.87$. From these results, it was suggested that the SDS-binding capacity is linearly proportional to the surface hydrophobicity of proteins. Therefore, the SDS-binding capacity can be used as a measure of protein hydrophobicity. The hydrophobic partition method (Shanbhag and Axelsson, 1975) and fluorescence probe method (Kato and Nakai, 1980) have been established to determine the surface hydrophobicity of proteins. However, these methods are greatly affected by a turbidity. Therefore, another method should be developed to measure the surface hydrophobicity of insoluble protein systems. The SDS-binding method seems to be suitable for the measurement of surface hydrophobicity of insoluble proteins. Since only free SDS extracted with $CHCl_3$ from the insoluble SDS–protein complex can be measured by this method, there is no demerit in measuring the surface hydrophobicity of insoluble proteins.

An attempt was made to determine the surface hydrophobicity of insoluble proteins using the SDS-binding method. Figure 6 shows the changes in the SDS-binding capacity of insoluble denatured ovalbumin during heat denaturation. The ovalbumin solution became turbid by

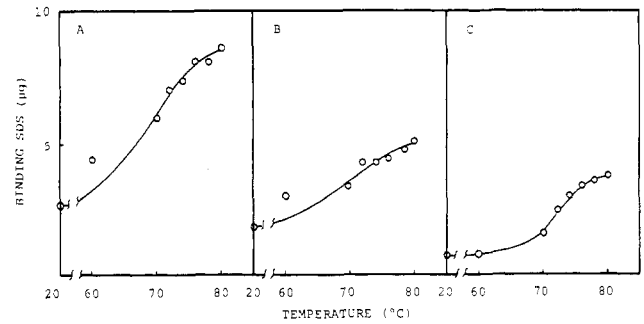

Figure 6. Changes in SDS-binding capacity of ovalbumin during heat denaturation at various pHs. (A) pH 4.5; (B) pH 5.8; (C) pH 7.4.

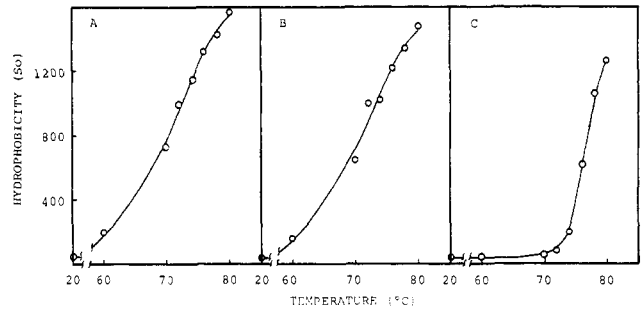

Figure 7. Changes in surface hydrophobicity of ovalbumin during heat denaturation at various pHs. (A) pH 4.5; (B) pH 5.8; (C) pH 7.4.

heating above 60 °C at acidic pH (Figure 6A,B). The SDS-binding capacity of insoluble denatured ovalbumin at pH 4.5 and pH 5.8 greatly increased with heating, compared to that of soluble denatured ovalbumin at pH 7.4. Changes in the surface hydrophobicity of ovalbumin were also investigated during heat denaturation at acidic pH by using the fluorometric method. As shown in Figure 7, large differences of the surface hydrophbicity were not observed between soluble denatured ovalbumins (C) and insoluble denatured ones (A and B), although the S_0 value of insoluble denatured ovalbumin (A) at 80 °C was 1.2 times that of soluble denatured ovalbumin (C) at 80 °C. On the other hand, large differences of SDS-binding capacity were observed between soluble and insoluble ovalbumins, as shown in Figure 6. The amount of binding SDS of insoluble ovalbumin (A) at 80 °C was more than twice that of soluble ovalbumin (C) at 80 °C. This may be due to the reason that the surface hydrophobicity (S_0) of insoluble ovalbumins is estimated to be smaller than true values because the fluorescence intensity of *cis*-parinaric acid is inhibited by turbidity. Therefore, the SDS-binding method may be superior to the fluorometric method in determining the surface hydrophobicity of insoluble proteins.

Registry No. SDS, 151-21-3; lysozyme, 9001-63-2; ribonuclease, 9001-99-4; trypsin, 9002-07-7; catalase, 9001-05-2; lipase, 9001-62-1.

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Preparation and Purification of Lactulose from Sweet Cheese Whey Ultrafiltrate

Kevin B. Hicks,* Donna L. Raupp, and Philip W. Smith

Several methods were developed for efficiently preparing lactulose from the lactose found in cheese whey ultrafiltration permeate. Lactose and organic acid content of this ultrafiltrate were first measured by high-performance liquid chromatography (HPLC). An amount of boric acid that was equimolar to the lactose content was added to the ultrafiltrate, the pH of the solution was adjusted to 11 with either triethylamine or sodium hydroxide, and then the solution was heated (70 °C) for a given interval. Either normal or concentrated whey ultrafiltrate (with lactose concentrations ranging from 4.4 to 17.7 g/100 mL) was used with the resulting yields of lactulose (measured by HPLC) exceeding 80%, based on the starting lactose content. Five different purification procedures were examined for the removal of catalysts, boric acid, and noncarbohydrate whey components from the reaction products. A combination of strong acid, adsorption, and boron selective ion-exchange resins was especially effective for this purpose. The lactulose syrups produced by these methods can be treated by the procedures given, to yield pure crystalline lactulose.

Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) is a synthetic ketose disaccharide that has several unique pharmaceutical applications (Bircher et al., 1966; Conn, 1978; Méndez and Olano, 1979). This useful sugar was originally synthesized by the isomerization of lactose in basic solution (Montgomery and Hudson, 1930). That process, and others of a similar nature, produces low yields of lactulose, which is difficult and expensive to refine. Most lactulose, therefore, is marketed as an impure syrup that contains varying amounts of related carbohydrates and their rearrangement products (Huhtanen et al., 1980). The high cost and low purity of these preparations have deterred the development of new food applications, even though this sugar has properties (high solubility, nondigestibility, and moderate sweetness) that make it an ideal candidate for certain limited food uses. In an attempt to make lactulose more available for research purposes, we recently (Hicks and Parrish, 1980) prepared pure, crystalline lactulose by isomerizing lactose in the presence of boric acid and base catalysts. This process produces the ketose in high yeilds when pure, refined α-lactose monohydrate is used as a starting material.

Recently, dairy processors have been using a process of ultrafiltration to recover valuable proteins from cheese whey. This ultrafiltration permeate, or ultrafiltrate, may be further refined by ion-exchange treatment and evaporation, to yield pure lactose (Delbeke, 1979). If the lactose in the whey ultrafiltrate could be directly converted to lactulose, however, the energy-intensive lactose refining step could be obviated. This paper, therefore, describes methods for the efficient synthesis and HPLC analysis of lactulose directly from either regular, concentrated, or deionized sweet whey ultrafiltrate. In addition, several methods for the purification and crystallization of these lactulose syrups, including a boron selective chromatographic process, will be described.

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118.

MATERIALS AND METHODS

Preparation and Analysis of Sweet Whey Ultrafiltrate. Fresh sweet whey from full cream cheddar cheese was strained from the vat outlet and fed to a 1200-L storage tank. The whey (at 38 °C) was immediately pumped through a DeLaval Model 340B clarifier to remove fines and a DeLaval Model 340B separator to remove cream, pasteurized at 72 °C for 15 s, and cooled to 46 °C in the regeneration section of a Chester Jensen high-temperature short-time pasteurizer. A sanitized 1200-L storage vat was used as a supply reservoir to feed a home-built Romicon ultrafiltration unit with two Model HF 26.5-45-XM50 membrane cartridges in parallel. The cartridges were operated at an optimum operating temperature of 49 °C and a pressure of less than 25 psi. After filtration of 115 kg of sweet whey 24 h^{-1} $cartridge^{-1}$, the unit was back-blushed, cleaned, and sanitized (200 ppm of sodium hypochlorite). Ultrafiltrate was collected, cooled to 4.5 °C, and then frozen. Prior to reaction, the whey ultrafiltrate was thawed, stirred at room temperature for 1 h, and then filtered through Whatman no. 2 paper. In some cases, the whey ultrafiltrate was then either deionized (Delbeke, 1979) or concentrated, prior to reaction. Total solids and ash values for the whey ultrafiltrates were determined by AOAC (1980) methods 16.032 and 16.035. The composition of a typical unconcentrated whey ultrafiltrate was ash 0.51%, solids 5.8%, and nitrogen 0.051%, pH, 6.3. Ultrafiltrates exhibited minor (ca. 10%) lot to lot variations in these values.

High-Performance Liquid Chromatography. For the determination of lactose and organic acids in whey ultrafiltrate, an HPLC system was used that consisted of a DuPont 870 pump module, a DuPont column compartment (65 °C), a Valco injector (20-μL loop), and a