The effect of carbohydrates on the quantitative determination of HMF was also examined. The experimental procedure, in this case, was as follows: A single sugar or a combination of carbohydrates were mixed with a known volume of TRI-SIL and left for 5 min at room temperature. To the mixture was then added a known amount of a benzene solution of HMF and after the appropriate waiting period the material was examined by gas chromatography. A typical chromatogram of a mixture containing several sugars and HMF is shown in Fig. 1. The retention times of various sugars, as compared to HMF, are shown in Table III. The retention times show that of the sugars tested only xylose and ribose interfere, but even in these two cases the peaks can easily be resolved. A chromatogram showing the level of interference by the two sugars is presented in Fig. 2.

Furfural has no hydroxyl group and, therefore, cannot form an ether derivative with the TRI-SIL reagent. Under the experimental conditions used furfural does not interfere with the HMF determination even when the concentration of furfural is three times that of HMF.

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Received April 11th, 1968

J. Chromatog., 36 (1968) 359–362

CHROM. 3609

Analytical fractionation of plant and animal proteins by gel electrofocusing

The technique known as isoelectric focusing or electrofocusing involves the migration of protein molecules to the regions of their isoelectric pH values in a pH gradient. In the original procedure[1,2], the pH gradient is produced in a specially designed column by applying a voltage to a mixture of carrier ampholytes stabilized in a sucrose density gradient. The result of the fractionation is determined by analysing fractions drained from the column. The present communication describes a simple procedure for the rapid analysis of small amounts of protein using the principle of isoelectric focusing. By performing the fractionation in tubes of polyacrylamide gel containing protein sample and carrier ampholytes, a number of samples can be analysed simultaneously in several hours, compared with several days for a single fractionation by the original procedure.

Experimental

The apparatus for gel electrofocusing was similar to that used for disc electrophoresis (DAVIS[3]). The polyacrylamide gel was set in glass tubes 5 mm in diameter and either 65 or 120 mm long. The tubes were inserted in holes (rimmed with rubber grommets) in the base of an upper electrode vessel and dipped into the electrolyte of a lower electrode vessel.

TABLE I

SOLUTIONS FOR POLYMERIZATION OF GELS FOR ISOELECTRIC FOCUSING

Catalyst solution		*Concentrated gel mixture*	
N,N,N′,N′-Tetramethylethylenediamine	1.0 ml	Catalyst solution	0.8 ml
Riboflavin	14 mg	Acrylamide solution	3.0 ml
Water	to 100 ml	Carrier ampholytes	0.3 ml
Acrylamide solution		*Gel mixture (one short tube)*	
N,N′-Methylenebisacrylamide	0.8 g	Concentrated gel mixture	0.5 ml
Acrylamide	30 g	Protein sample	30–300 μg
Water	to 100 ml	Water	to 1.5 ml

The polyacrylamide gel was prepared by mixing the solutions out in Table I. Gel tubes were filled to within 2 mm of the top. The gel mixture was overlayered with water and photopolymerized by exposure to a fluorescent light for 30 min. (Alternatively, the gel can be polymerized chemically by replacing the riboflavin with 0.7 mg ammonium persulphate per ml gel.) When using gluten proteins, urea was used in the sample to give a concentration of 2 *M* in the gel. Carrier ampholytes (40 % solution) were obtained from LKB-Produkter AB, Sweden.

Fractionation was performed at room temperature. Eight gel tubes were placed in the upper electrode vessel (anode) which was filled with 250 ml 0.2 % sulphuric acid. The lower vessel (cathode) contained 250 ml 0.4 % ethanolamine. A current of 1 mA per tube for long gels or 2 mA per tube for short gels was maintained by gradually increasing the voltage up to 350 V. This voltage was maintained to the end of the run. The electric field was applied for a total of 3 h for short gels or 5 h for long gels.

Gels were removed from the tubes by rimming with a hypodermic needle with water running through it. Protein fractions in the gels were visualized as white precipitation bands by immersing the gels in 5 % trichloroacetic acid. Since the carrier ampholytes stain strongly with protein dyes such as amido black, the gels must be washed repeatedly in 5 % trichloroacetic acid to remove the ampholytes. The washed gels were stained for one hour in amido black (1 % in 7 % acetic acid) and destained by washing in 7 % acetic acid.

The course of the pH gradient in gels was determined by cutting 5 mm sections from an unstained gel run simultaneously with stained gels, soaking each section in 2 ml water for several hours and measuring the pH of the extracts. The pH gradient was reproducible for gels run simultaneously.

Water-soluble proteins of wheat flour (variety Gabo) were extracted with sodium pyrophosphate (0.01 *M*, pH 7) after an initial extraction with water-saturated butanol (WRIGLEY[4]). Gluten proteins were extracted from the same flour sample with

A_1 and A_2 (and at higher loadings A_3). Dephosphorylated ovalbumin showed two bands, dephosphorylated A_1 (with a similar isoelectric point to A_2) and A_3. PERLMANN[7] reported that the isoelectric points of A_1, A_2 and A_3 are 4.58, 4.65 and 4.74 (0.1 ionic strength). Fig. 2e shows the separation of P_1 and P_2 from plakalbumin (isoelectric points 4.72 and 4.80, respectively). The isoelectric points of these proteins separated by gel electrofocusing (see pH curve, Fig. 2) correspond approximately to the values reported by PERLMANN. Although gel electrofocusing in 5 mm tubes does not permit accurate determination of the isoelectric point, the technique serves as a guide in establishing the most suitable conditions for preparative fractionation in sucrose density gradient or in larger gels.

Preparation of fractions and more accurate determination of isoelectric point is possible by performing gel electrofocusing in a slab of gel instead of in columns. For the slab technique protein fractions are located by staining a test strip cut from the side of the gel. Electrofocusing in a gel would be more suitable than electrofocusing in a density gradient for the fractionation of proteins that tend to precipitate at the isoelectric point.

Gel electrofocusing can be used for analysing very small amounts of protein in low concentrations. Using the gel formulations in Table I, a minimum concentration of 0.002 % protein can be used. When using samples of higher concentration, an alternative method of application is to layer the sample solution (containing 10 % sucrose) on top of the gel and under a protecting layer of carrier ampholyte solution (containing 5 % sucrose).

Note added in proof

At about the same time as preliminary reports[8,9] of this work were presented, variations of the technique were described by DALE AND LATNER[10] and LEABACK AND RUTTER[11].

Acknowledgement

Thanks are due to Mrs. C. TRENOUTH for skilful technical assistance.

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Received May 20th, 1968