CELLULOSE DIALYSIS MEMBRANES: A POSSIBLE SOURCE OF PEPTIDE CONTAMINATION

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
RICHARD EMMETT McDONALD
1973

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ABSTRACT

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PEPTIDE CONTAMINATION

By

Richard Emmett McDonald

Previous research has indicated that peptides were associated with isolated Beta-lactoglobulin (β-Lg) preparations. The purpose of this study was to isolate and characterize these peptides.

Peptides were isolated from 4X recrystallized β-Lg by electrodialysis. The peptide mixture was separated into eight peptide fractions by two dimensional peptide mapping on MN 300 cellulose thin-layer chromatography plates.

Gel filtration chromatography indicated the molecular weight of the peptides to be between 700 and 5,000 Daltons. Two of the peptides were shown to have carbohydrate associated with them.

Results from amino acid analyses of β-Lg and (E) β-Lg gave the first indication that the peptides were not associated to β-Lg. Electrodialysis was run for 48 hours using deionized water in place of β-Lg. The same eight peptides originally believed to be associated with β-Lg were present when two dimensional peptide mapping was performed. This confirmed that the peptides previously reported to be associated to β-Lg were an artifact of the isolation procedure.

Further studies showed that the peptides were also released during static dialysis. This indicated that peptides were released from cellulose membranes during normal dialysis procedures such as the desalting of proteins.

Micro-Kjeldahl determinations indicated that cellulose membranes contain about 0.15% protein. Since peptides are present in such small quantities, they should not affect the properties of major protein fractions. However, it is important to recognize the presence of these peptides if minor protein fractions are being studied.

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Richard Emmett McDonald

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science
and Human Nutrition

1973

ACKNOWLEDGMENTS

The author expresses sincere gratitude to Dr. G. A. Leveille, Chairman of the Department of Food Science, and to Dr. J. R. Kirk, Assistant Professor of Food Science, for their advice, counsel, and encouragement.

Grateful acknowledgment is due to the Department of Food Science, Michigan State University, for the facilities and funds which made this research possible.

The author expresses his sincere appreciation to his understanding and devoted wife, Jo Anne, who has helped in far too many ways to enumerate.

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INTRODUCTION

Beta-lactoglobulin comprises seven to twelve percent of the total proteins found in milk. The properties of this protein have been extensively studied for many years, but its biological function remains in doubt.

One of its functions may be to serve as a vitamin carrier in milk. In vitro, β-Lg has been found to have a high vitamin B_{12} binding capacity, although the mechanism of this binding has not yet been shown.

Early research indicated that the mechanism for vitamin B_{12} binding was related to peptides that had been reported to be electrostatically associated to β-Lg. However, Kirk et al. (1972) showed no involvement of peptides in vitamin B_{12} binding. The function of these peptides, therefore, was still unknown.

This study was initiated to determine the characteristics and function of peptides believed to be associated with β-Lg.

LITERATURE REVIEW

Beta-Lactoglobulin (β-Lg)

β-Lg is a major whey protein comprising seven to twelve percent of the total protein in bovine milk. Palmer (1934) first isolated and crystallized β-Lg by salt fractionation from skim milk. Aschaffenburg and Drewry (1955 and 1957b) found that β-Lg contained two genetic variants designated A and B. They found that bovine milk could contain either or both of these variants. Bell (1962) later showed the presence of a third genetic variant, β-Lg C, in the milk of Jersey cows.

Grosclaude *et al.* (1966) found a fourth genetic variant that was designated β-Lg D in Montbeliarde cattle in France. Another genetic variant, $\beta\text{-Lg}_{\text{Droughtmaster}}$, has been reported by Bell *et al.* (1966) to be present in the milk of Australian Droughtmaster beef cattle. This variant has the same amino acid composition as the bovine A variant, but has a carbohydrate moiety attached to it.

The molecular weight of β-Lg has been reported by Pedersen (1936), Bull and Currie (1946), Ogston and Tilley (1955), Green and Aschaffenburg (1959), Wirtz et al. (1964), and Aschaffenburg et al. (1965) to range between 35,000 and 37,000 Daltons. Generally, the molecular weight of bovine β-Lg is considered as 36,000 Daltons. A complete amino acid sequence of β-Lg AB was recently proposed by Braunitzer et al. (1972).

Bull (1946) and Bull and Currie (1946) first showed that native β-Lg is a molecular dimer composed of two 18,000 Dalton polypeptides. These two subunits form a stable dimer as a result of hydrophobic interactions between a pH of 4.5 and 7.5 (Timasheff, 1964).

β-Lg A has been shown to contain two more aspartic acid and two more valine residues than β-Lg B; whereas, β-Lg B has two more glycine and two more alanine residues than β-Lg A (Kalan et al., 1964). It is also shown by Kalan et al. (1965) that β-Lg C has two more residues of histidine and two fewer glutamine residues than β-Lg B per 36,000 molecular weight.

β-Lg has been shown to undergo conformational changes both above and below its isoelectric point of 5.2.

Timasheff and Townend (1961) showed that each genetic variant of β-Lg undergoes a rapid monomer-dimer equilibrium, with increasing dissociation as the pH is lowered below 3.5. At pH 4.5 β-Lg forms a tetramer having a molecular weight of 144,000 Daltons, which is composed of either an aggregate of homozygous or heterozygous genetic variants of β-Lg (Kumosinski and Timasheff, 1966). β-Lg also undergoes conformational changes above its isoelectric point. β-Lg dimers are reversibly dissociated into their monomeric subunits at a pH above 8 (Georges et al., 1962).

Peptides

Gizis et al. (1965), using electrodialysis of skim milk, reported the isolation of vitamin B_{12} binding peptides. Using a similar procedure, Dorris (1968) isolated two peptides from β-Lg and concluded that these peptides were partially responsible for vitamin B_{12} binding to β-Lg in milk. He also reported peptides to be released by α-lactalbumin and an intrinsic factor preparation.

Kirk (1971) reported three peptides to be electrostatically associated to β-Lg, but found no correlation between these peptides and vitamin B_{12} binding to β-Lg.

Cellulose Dialysis Membranes

Dialysis requires that the membrane separating the liquids permit diffusional exchange between the molecular species present, while effectively preventing any convective exchange between the two solutions. Dialysis was first discovered by Thomas Graham in 1861, who successfully used dialysis to separate "crystalloids" (low molecular weight substances) from colloids (Leonard, 1965).

Cellulose membranes have been extensively used for dialysis for several years. These membranes are formed from regenerated cellulose and have in the past been referred to as cellophane, Visking cellulose, or dialysis tubing (Weber et al., 1970). The raw materials necessary for the production of this regenerated cellulose are wood pulp, caustic soda, and carbon disulfide. According to Sineath and Pavelchek (1971), caustic soda is added to wood pulp and the wood is then shredded before carbon disulfide is added. This mixture then goes through several mixing tanks before the solution is filtered to yield what is called the viscose solution. Viscose is a colloidal suspension of cellulose xanthate in sodium hydroxide solution. The viscose is deaerated, given a final

filtration, and then transferred to a casting machine for casting into a continuous gel sheet. This sheet of cellulose xanthate is then decomposed by sulfuric acid and sodium sulfate to form a regenerated cellulose sheet that is completely insoluble in water. The regenerated cellulose film is passed through several tanks of hot water to drive off the carbon disulfide and hydrogen sulfide, wash sulfuric acid from the film surface, and wash out sulfuric acid and sodium sulfate that may be dissolved in the film. Remaining sulfur compounds that may be left in the film are removed by treating the film with a hot sodium hydroxide solution. The film is then bleached in a sodium hypochlorite or peroxide solution and plasticized with glycerol or propylene glycol.

According to Craig (1967), the porosity of wet cellulose casing can be adjusted by using procedures such as mechanical stretching, acetylation, or zinc chloride treatment.

Craig (1967) also reported that the impurities in cellulose dialysis films are usually leached out during repeated soaking in .01N acetic acid; but, for careful work in which metals must be excluded, the membrane should be soaked in a solution of ethylenediaminetetraacetic acid

(EDTA). McPhie (1971) reported the use of a much more extensive treatment by soaking the membrane in ethyl alcohol, sodium bicarbonate, and EDTA solutions.

Crevasse and Pearson (1969) have reported the presence of "nitrogenous ninhydrin positive" contaminants in dialysis tubing, even after careful washing in 10% sodium bicarbonate and .05% acetic acid.

EXPERIMENTAL PROCEDURE

Preparative Methods

Beta-Lactoglobulin: Isolation

β-Lg was isolated essentially as described by Aschaffenburg and Drewry (1957a). See appendix for details.

Beta-Lactoglobulin: Recrystallization

See appendix for details.

Chemical Methods

Nitrogen

Percent nitrogen was determined using a micro-Kjeldahl technique. See appendix for details.

Amino Acid Analysis

Amino acid analyses were carried out on 22 hour hydrolysates of the β-Lg, electrodialyzed β-Lg [(E) β-Lg], and the isolated peptide mixture employing a Beckman Amino Acid Analyzer (Model 120C) according to the method of Moore, Speckman, and Stein (1958). See appendix for details.

Treatment of Membranes

Cellulose membranes used in this research were obtained from Union Carbide Corporation. All membranes used were pretreated to eliminate impurities. Initially, membranes were treated by boiling them in a dilute EDTA solution and thoroughly rinsing them in deionized water prior to use.

A more extensive pretreatment of the membranes was also conducted according to the procedure of McPhie (1971). This procedure consisted of simmering the membranes in a 50% solution of ethanol for one hour, then repeating this process. The membranes were then soaked in dilute sodium bicarbonate and dilute EDTA solutions for one hour each,

and stored in 0.02% sodium azide at 4°C. Prior to use the membranes were exhaustively washed in deionized water.

Physical Methods

Method of Electrodialysis

The electrodialysis procedure was a modification of the method reported by Kirk (1971). Electrodialysis was performed in a cell consisting of three parts: 1. a glass cylinder (45 cm. long and 4.8 cm. internal diameter) to hold the dialysis water, 2. a U-shaped glass rod to form an internal frame for the dialysis membrane, and 3. two platinum foil electrodes.

A cellulose membrane treated as previously described was knotted at one end and slipped over the U-shaped glass frame. This frame served as a support for the platinum electrode, as well as the framework for the membrane.

The membrane to be used for electrodialysis was prerun by filling the membrane with deionized water and inserting it into the glass cylinder. The cylinder was also filled with deionized water, and the entire cell

placed in an ice bath to ensure proper cooling. A power source was connected to the platinum electrodes, with the electrode in the water outside the membrane serving as the cathode and the electrode in the water inside the membrane serving as the anode. This system was then run for two hours at 200 volts, while changing both the water outside the membrane (dialysate) and water inside the membrane (diffusate) every 30 minutes.

A suspension consisting of three grams of β-Lg in 125 ml. of deionized water was then poured into the dialysis membrane and the membrane inserted inside the glass cylinder filled with deionized water. The β-Lg was kept in a uniform suspension by intermittently bubbling nitrogen into the bottom of the dialysis membrane through a glass rod. Electrodialysis was carried out at 200 volts.

Initially, there was little amperage through the cell. As charged particles began diffusing through the membrane, the current increased and reached a maximum value, at which time the dialysate water was changed and electrodialysis was continued. The current maximum was due to a charge equilibrium across the membrane. Thus, it was necessary to change the dialysate whenever the amperage

reached a maximum. The first two dialysate changes were discarded because it was felt they might contain ionic impurities from either the β-Lg or the membrane. Other dialysate fractions were collected until little amperage could be detected in the system. This usually required from nine to twelve hours, with a change in the dialysate approximately every hour.

The electrodialysate was concentrated in treated cellulose membranes by pervaporation, shell-frozen and lyophilized.

Polyacrylamide Gel Electrophoresis

Discontinuous polyacrylamide gel electrophoresis was performed by a modification of the procedure described by Melachouris (1969). See appendix for details.

Staining Polyacrylamide Gels

See appendix for details.

Preparation of Thin-Layer Plates

Thin-layer cellulose plates were prepared by suspending 15 g. of MN 300 cellulose in 90 ml. of deionized water and mixing the suspension in a Waring Blender. The slurried cellulose was applied to 20 x 20 cm. glass plates 500 microns thick, using a Desaga Brinkman thin-layer spreader. The plates were then air dried for 12 hours.

Silica gel G thin-layer plates were prepared by mixing 30 g. of silica gel G with 60 ml. of deionized water. The slurried silica gel was applied to 20 x 20 cm. glass plates 250 microns thick using a Desaga Brinkman thin-layer spreader. The plates were allowed to air dry for 30 minutes and activated at 110°C for 20 minutes.

High Voltage Thin-Layer Electrophoresis

High voltage thin-layer electrophoresis (HVTLE) was used both as an analytical and preparative procedure in the characterization and isolation of the peptides. Thin-layer cellulose plates were used for all HVTLE work.

Cellulose thin-layer plates were spotted with 15.0 µl. of a 0.1% peptide solution using a Cordis disposable

microapplicator and dried thoroughly. The plates were placed in a Reco Model E-800-2 water cooled electrophoretic migration chamber and wicked to the buffer tanks with filter paper. Two different buffers were used in the buffer tanks during the course of this research: 1. pyridine-acetic acid-water (1:10:189), pH 3.5; and 2. pyridine-acetic acid-water (5:9:986), pH 4.5. The thin-layer plates were sprayed with the same buffer used in the electrode buffer tanks. A field strength of 40 V/cm. was applied to the thin-layer plates for 20 minutes. The plates were then dried in an oven at 90°C.

Location of the peptides was accomplished by spraying the plates with ninhydrin reagent (1 g. ninhydrin, 700 ml. absolute ethanol, 29 ml. of 2, 4, 6 - trimethyl-pyridine, and 210 ml. glacial acetic acid), and drying at 90°C until the peptides became visible.

Preparative HVTLE plates were run to isolate four of the peptide components. This procedure was similar to the procedure for analytical plates described above. However, instead of spotting the plate, Cordis disposable micro-applicators were used to streak approximately 400 µl. of a 0.1% peptide solution across the entire width of the plate.

The plates were then placed in the electrophoretic migration chamber and electrophoresis was conducted for 20 minutes at pH 4.5. After drying at 90°C, location of the peptides was accomplished by spraying narrow strips (1.5 cm. wide) on the plate edges in the direction of migration with ninhydrin reagent, as described above. A ruler was used to mark off the areas containing the individual peptide streaks and the cellulose layer was scraped from these areas. The peptides were eluted from the MN 300 cellulose with 5% acetic acid (Kirk, 1971).

Thin-Layer Chromatography

MN 300 cellulose thin-layer plates were spotted with 5.0 µl. of a 0.1% peptide solution and dried thoroughly. The plates were developed in a solvent tank containing isopropanol-formic acid-water (40:2:10) solvent. They were taken out and dried when the solvent front approached the top of the plates. The plates were sprayed with ninhydrin reagent and dried in an oven at 90°C until the peptide spots became visible.

The preparative TLC plates were treated similarly, except that as in preparative HVTLE, Cordis disposable

microapplicators were used to apply 400 µl. of a 0.1% peptide solution across the width of the plates. TLC was performed as above. After the plates were dried, location of the peptides was accomplished by spraying narrow strips with ninhydrin, as described under preparative HVTLE. The cellulose containing each isolated peptide was scraped from the plates and the peptides were recovered by elution with 5% acetic acid.

Two Dimensional Peptide Mapping

Two dimensional peptide mapping was used as an analytical technique to determine how many peptide components were present in the peptide mixture. Cellulose thin-layer plates were spotted with 5.0 µl. of a 0.1% peptide solution and dried thoroughly. High voltage thin-layer electrophoresis was then performed using a buffer of pyridine-acetic acid-water (5:9:986), pH 4.5, at a field strength of 40 V/cm. for 20 minutes. The plates were then dried in an oven at 90°C and cooled to room temperature.

The second dimension of peptide mapping was carried out using thin-layer ascending chromatography. The thin-

layer plates were placed in a thin-layer solvent tank containing isopropanol-formic acid-water (40:2:10). When the solvent front approached the top of the plates, they were taken out, dried, and sprayed with ninhydrin reagent to detect the peptide spots.

Identification of Glycopeptides

Thin-layer silica gel G plates were used to identify glycopeptides according to the procedure of Moczar (1973). The isolated peptides were spotted on the plates with capillary tubes and developed in a thin-layer tank with butanol-acetic acid-water (80:20:20) as the migrating solvent. After the solvent front had migrated near the top of the plates, they were put into an oven at 90°C until the peptide spots became visible. The plates were resprayed with orcinol-sulfuric acid reagent (0.1% orcinol in 2N H_2SO_4) and the color developed in a 100°C oven for 10 to 15 minutes. A positive color reaction indicated the presence of a glycopeptide. The development of the color is not influenced by the previous treatment with ninhydrin reagent.

Gel Filtration Chromatography

Gel filtration chromatography using Sephadex G-25 and G-10 was used in this study. An excess of boiled buffer (0.1N ammonium acetate) was added to the Sephadex to be rehydrated. A uniform suspension of gel was made by carefully stirring the Sephadex into the hot buffer with a glass rod. When cool, the excess buffer was decanted off and another solution of boiled buffer was added to the Sephadex. This procedure was repeated at least three times. After rehydration was complete, a slurry of the Sephadex gel was made by mixing an equal volume of buffer and swelled Sephadex. The slurry was deaerated under vacuum and carefully poured into a Sephadex chromatographic column. After about 5 to 10 minutes, the column outlet was opened to allow complete packing of the gel bed.

The column was equilibrated with two column volumes of 0.1N ammonium acetate buffer prior to use. The void volume of the columns was determined using Blue Dextran 2000. The eluant coming from the column was monitored at 254 nm. using an Isco UV column monitor.

RESULTS AND DISCUSSION

Nitrogen Content of Beta-Lactoglobulin

Nitrogen analysis of the isolated β-Lg by the micro-Kjeldahl method gave a value of 15.91%. This compares to the literature value of 15.6% reported by Larson and Jenness (1955). A conversion factor of 6.25 was used to determine the percent protein.

Gel Electrophoresis of Beta-Lactoglobulin

Vertical polyacrylamide gel electropherograms of the isolated β-Lg and (E)β-Lg are shown in Figure 1. Bands characteristic of β-Lg A and B, according to Melachouris (1969), were present in both β-Lg and (E)β-Lg. No contaminating proteins or peptides were present in detectable quantities in the β-Lg preparations using this technique. It was also shown that the electrophoretic

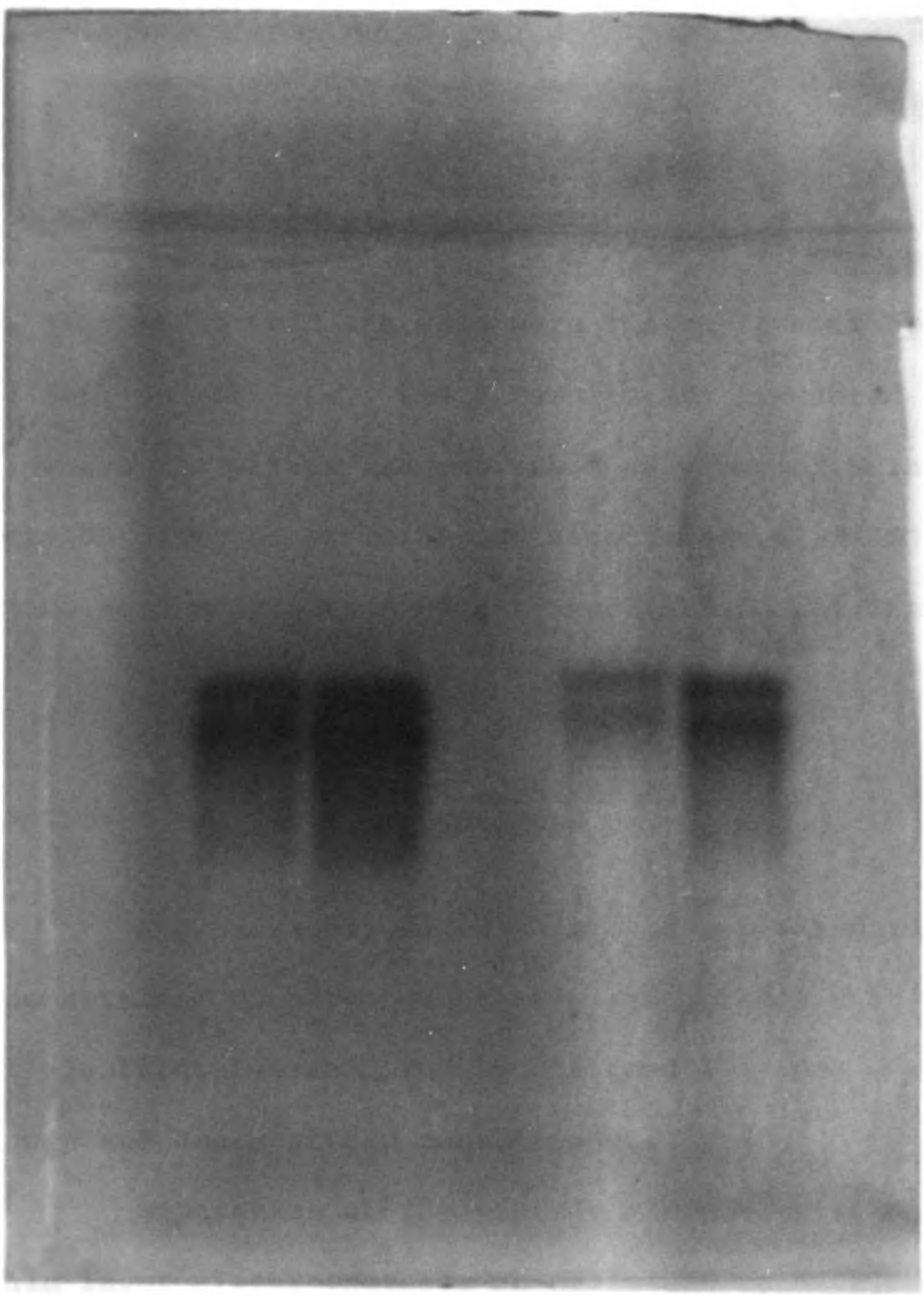

Figure 1.--Polyacrylamide gel electropherogram of β-Lg and (E)β-Lg at pH 8.3. Left to right: slot 1 1% β-Lg, slot 2 2% β-Lg, slot 3 1% (E)β-Lg, slot 4 2% (E)β-Lg.

mobility of β-Lg does not change significantly after electrodialysis.

Amino Acid Analysis of Beta-Lactoglobulin

Amino acid analyses were run on 22 hour hydrolysates of β-Lg and (E)β-Lg. These results are presented in Table 1, where the values are expressed as grams of amino acid per 100 grams of protein. No significant difference in amino acid composition of β-Lg and (E)β-Lg was detected.

Electrophoretic Mobility of Peptides

Approximately 30 to 40 mg. of peptide material was obtained after concentrating and lyophilizing 4,000 ml. of electrodialysate. The lyophilized peptides were light brown and had a sticky consistency.

Separation of the peptide mixture using high voltage thin-layer electrophoresis on cellulose thin-layer plates at pH 3.5 is shown in Figure 2. Four peptides were present. Three of these peptides exhibited the same mobilities under

TABLE 1.--Amino acid composition of beta-lactoglobulin and electrodialyzed beta-lactoglobulin

Amino Acid Residue	g amino acid residues/100 g protein βLg	(E) βLg
LYS	10.87	11.23
HIS	2.03	1.76
ARG	2.58	2.57
ASP	10.53	10.71
THR	4.40	4.41
SER	3.00	3.16
GLU	17.98	18.85
PRO	4.58	4.61
GLY	1.09	1.20
ALA	5.90	6.07
CYS	----	----
VAL	5.60	5.25
MTH	----	----
I-LEU	6.53	4.88
LEU	14.29	14.30
TYR	3.76	3.57
PHEN	3.06	3.34
TRYP	----	----
TOTAL*	100.00	100.00

*Total g amino acid residues/100 g protein

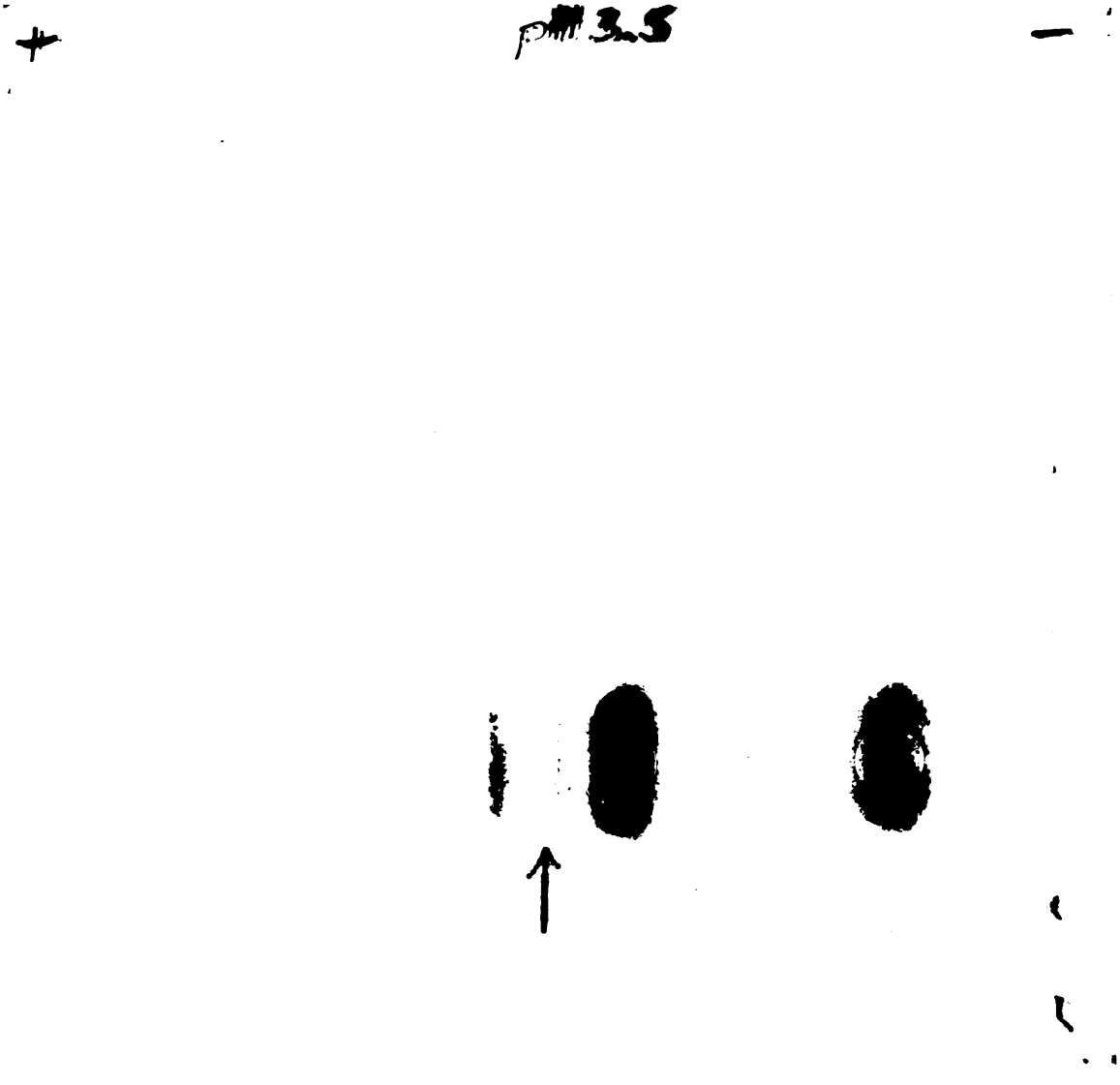

Figure 2.--Thin-layer high voltage electropherogram of the peptide mixture electrodialyzed from β-Lg. Support: MN 300 cellulose. Buffer: pyridine-acetic acid-water, pH 3.5.

these conditions as the three peptides reported by Kirk et al. (1972). The peptide that remained at the origin had previously been overlooked, probably due to the fact that it was present in very small concentrations. This peptide was named the negative 1 (neg 1) peptide. The other three peptides have already been named by Kirk et al. (1972) as follows: 1. the peptide migrating toward the anode was called the negative (neg) peptide, 2. the peptide migrating slightly towards the cathode was called the positive 2 (pos 2) peptide, and 3. the peptide migrating the farthest towards the cathode was designated the positive 3 (pos 3) peptide.

The mobilities of these peptides were also checked using HVTLE at pH 4.5. The same four peptides were evident, but they showed slightly different mobilities (Figure 3). The neg 1 peptide no longer stayed at the origin, but migrated toward the anode.

Two Dimensional Peptide Mapping

After failing to obtain homogeneous peptide fractions by ion exchange chromatography using SP(C-25)

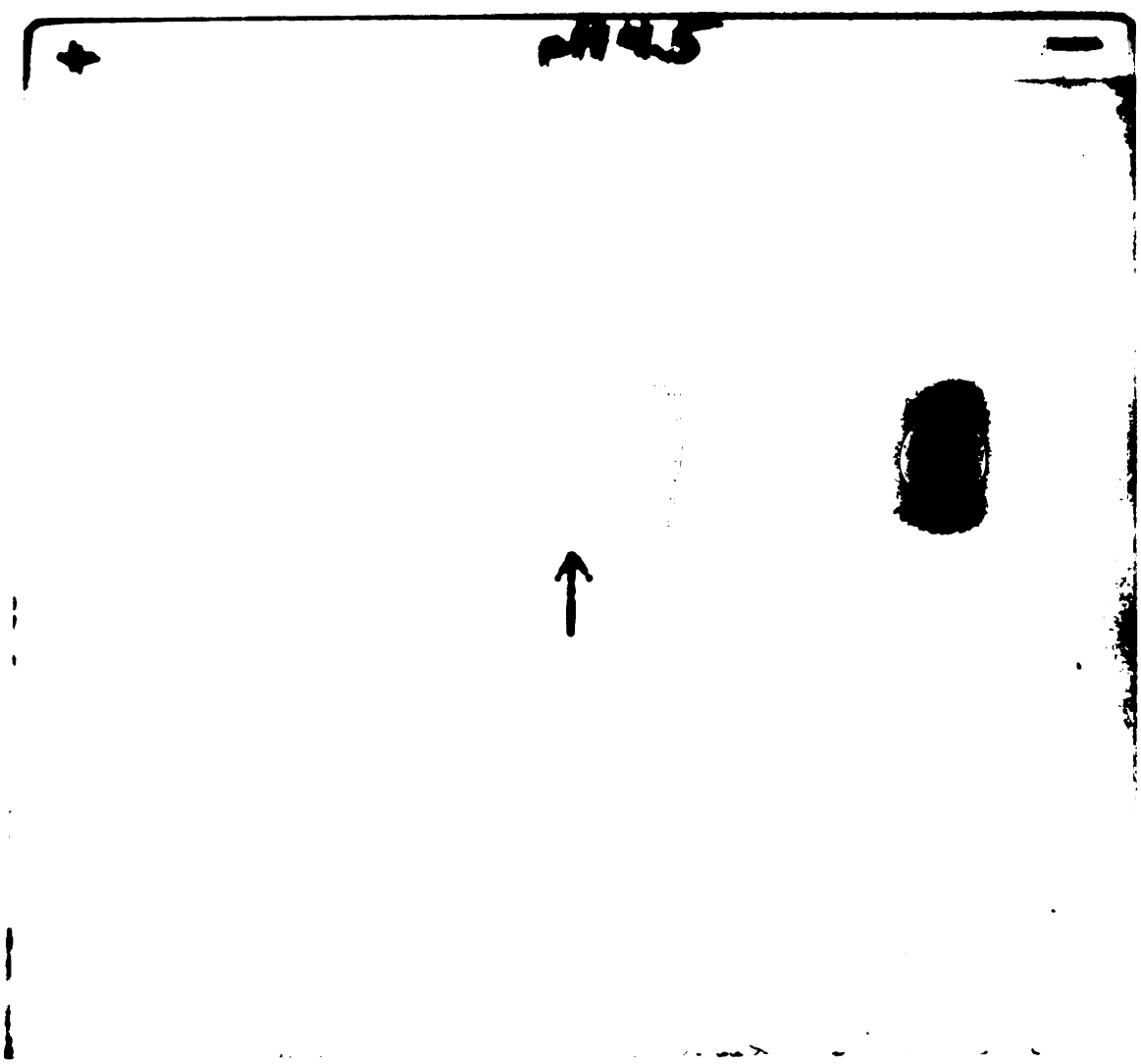

Figure 3.--Thin-layer high voltage electropherogram of the peptide mixture electrodialyzed from β-Lg. Support: MN 300 cellulose. Buffer: pyridine-acetic acid-water, pH 4.5.

Sephadex, it was decided that two dimensional peptide mapping should be done to check the homogeneity of each peptide fraction. The first dimension consisted of HVTLE at pH 4.5, and the second dimension ascending thin-layer chromatography with isopropanol-formic acid-water (40:2:10) as the migrating solvent (Figure 4). Eight peptides were observed. The original pos 2 peptide was shown to contain five peptide components. The additional five peptides were designated the pos 2-1, pos 2-2, pos 2-3, pos 2-4, and pos 2-5 peptides, with R_f values of 0.36, 0.44, 0.56, 0.71, and 0.86, respectively.

Isolation and Recovery of Peptides

Recovery of all eight peptides was accomplished by preparative HVTLE and ascending thin-layer chromatography techniques, as described in the experimental procedure section. The peptide mixture was first separated into four components (neg, neg 1, pos 2, and pos 3) using preparative HVTLE. Homogeneous isolates of the neg, neg 1, and pos 3 peptides were obtained by this procedure. The pos 2 peptide, which contained five components, was then streaked

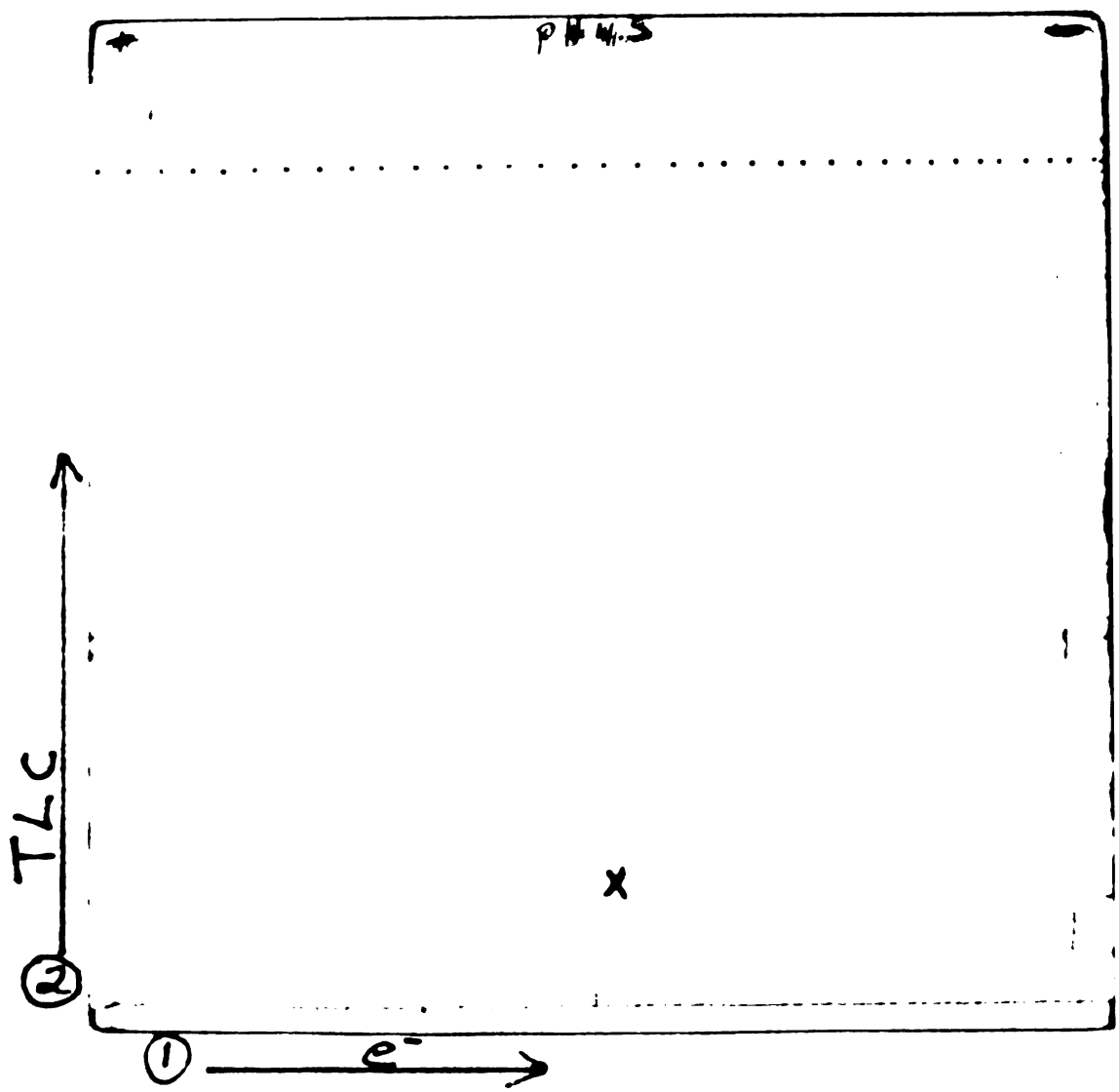

Figure 4.--Two dimensional peptide map of the peptide mixture electrodialyzed from β-Lg. Support: MN 300 cellulose. Electrophoresis: pyridine-acetic acid-water, pH 4.5. Chromatography: isopropanol-formic acid-water (40:2:10).

on cellulose thin-layer plates and separated using ascending thin-layer chromatography.

Cellulose containing each peptide was recovered from the HVTLE and TLC plates following separation and packed in a small glass column (1 x 10 cm.). The peptides were recovered from the cellulose by elution with 5% acetic acid solution. This procedure gave quantitative recovery of the peptides. However, impurities which were soluble in 5% acetic acid were eluted with the peptides. The 5% acetic acid solution containing both the peptide and impurity was lyophilized. A solvent mixture of isopropanol-formic acid-water (40:2:10) was then added to the lyophilized sample. Since all the peptides were completely soluble in this solvent and the impurity was completely insoluble, the impurity could be easily removed by centrifugation.

Molecular Weight of Peptides

Gel filtration chromatography was used to approximate the molecular weight of the peptides. The peptide mixture was passed over a G-10 Sephadex column (exclusion

limit 700) with a void volume of 21.0 ml. (Figure 5). All eight peptides were recovered in the void volume from the column, indicating that the peptides had a molecular weight of at least 700 Daltons. A second peak with an elution volume (V_e) of 44.5 ml. was eluted shortly after the peptides, but it did not exhibit a positive ninhydrin reaction.

A mixture of three amino acids, tyrosine, phenylalanine, and tryptophan was also passed over this same G-10 Sephadex column. Elution volumes of 40.0 ml., 47.0 ml., and 88.0 ml. were obtained (Figure 6). This helped to confirm the fact that the eight peptides are not amino acids.

Gel filtration chromatography of the peptide mixture over G-25 Sephadex (exclusion limit 5,000 and void volume 79 ml.) resulted in three peaks (Figure 7). The first two peaks which had elution volumes of 127 and 145 ml. contained all eight peptides. The third peak which had an elution volume of 185 ml. contained no peptides or amino acids. When a phenylalanine sample was passed over the same column, a peak having an elution volume of 182 ml. was observed (Figure 8).

Figure 5.--Elution pattern of electrodialyzed peptides from Sephadex G-10 column with 0.1N NH_4OAc. Peak 1-peptide mixture. Peak 2-salts and other impurities. Flow rate 0.5 ml. per minute.

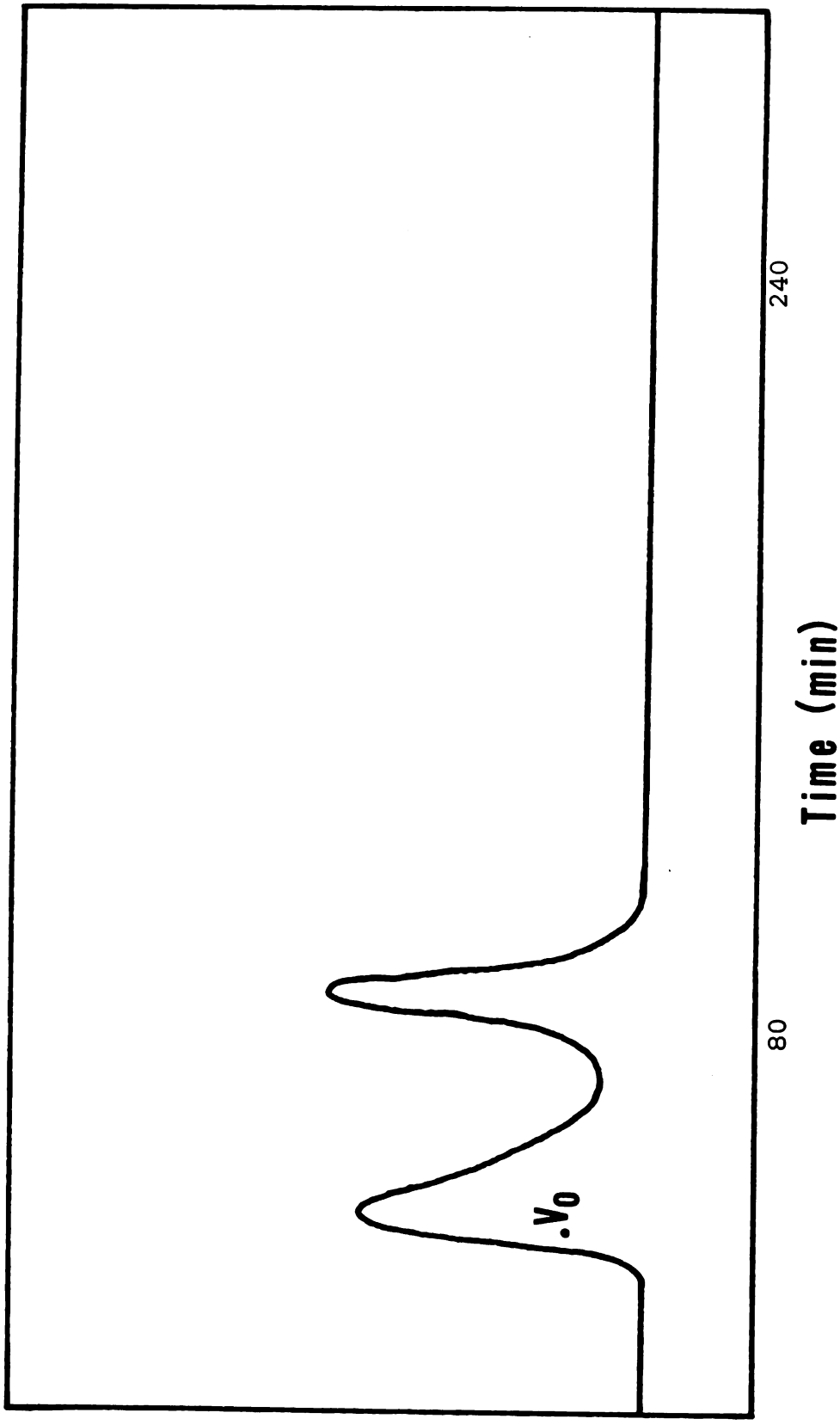

Figure 5.

Figure 6.--Elution pattern of tyrosine, phenylalanine and tryptophan from Sephadex G-10 column with 0.1N NH_4OAc. Flow rate 0.5 ml. per minute.

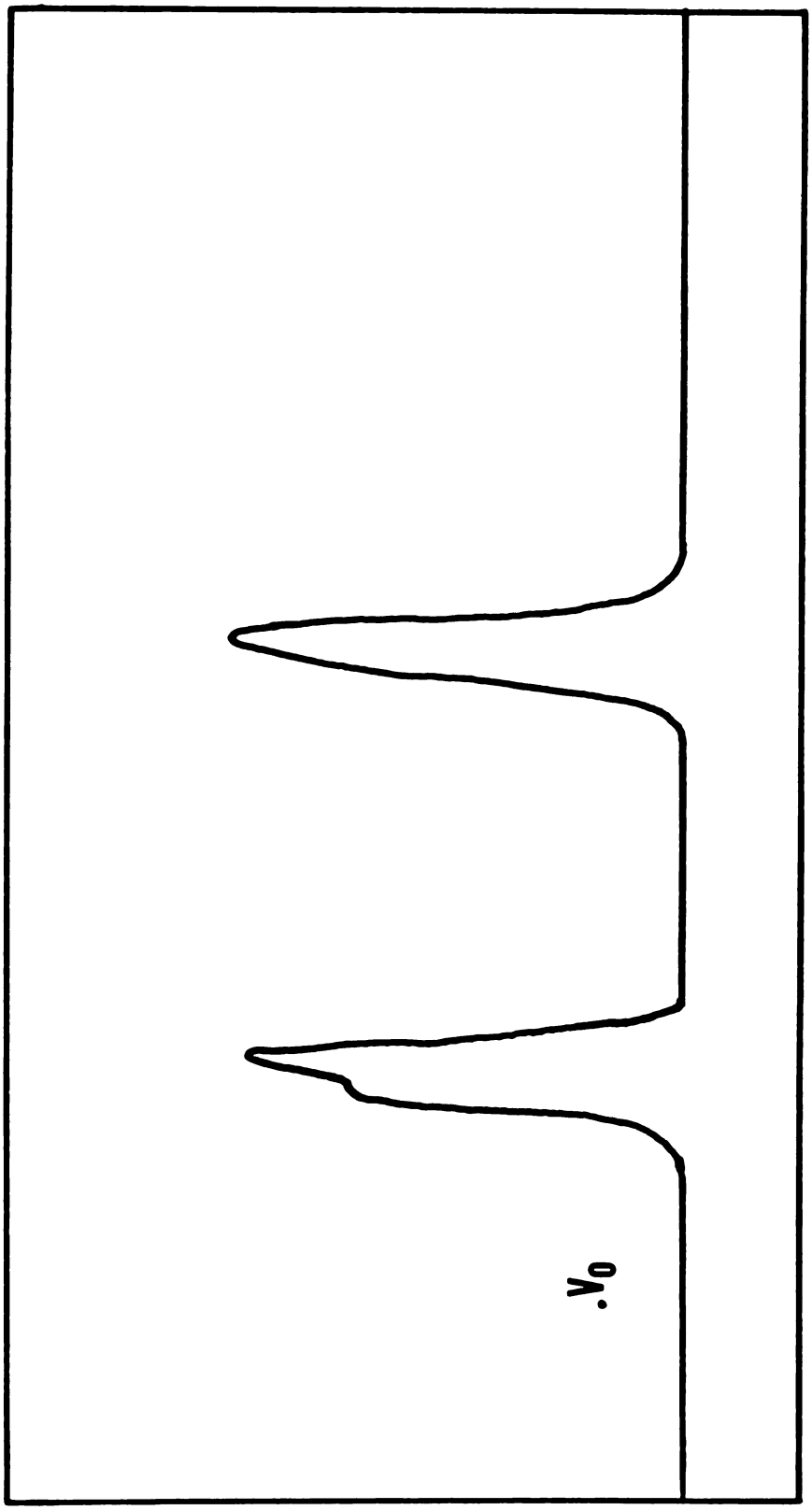

Figure 6.

Figure 7.--Elution pattern of electrodialyzed peptides from Sephadex G-25 column with 0.1N NH_4OAc. Peak 1 and 2 - peptide mixture. Peak 3 - salts and other impurities. Flow rate 1.0 ml. per minute.

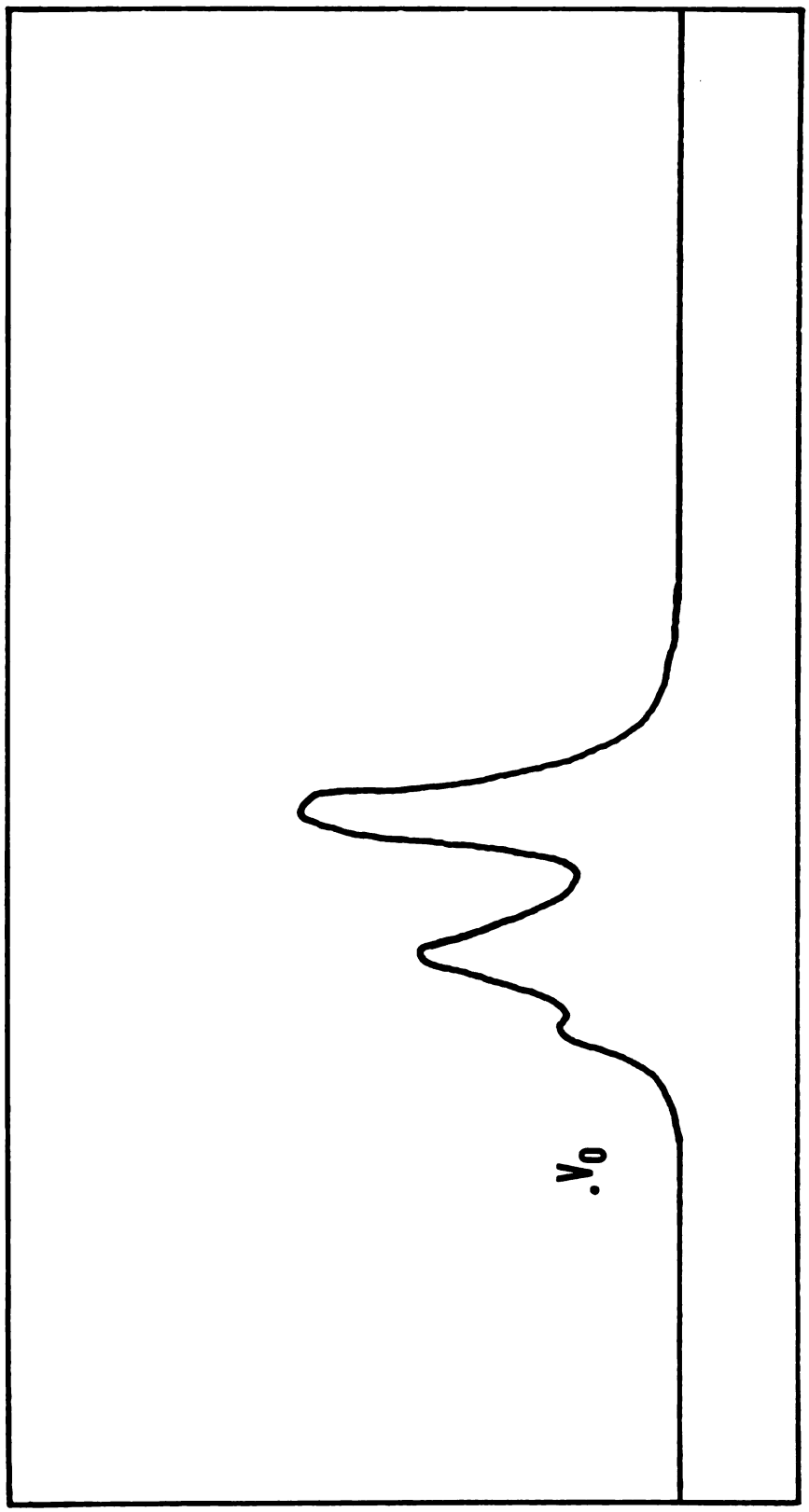

Figure 7.

Figure 8.--Elution pattern of phenylalanine from Sephadex G-25 column with 0.1N NH_4OAc. Flow rate 1.0 ml. per minute.

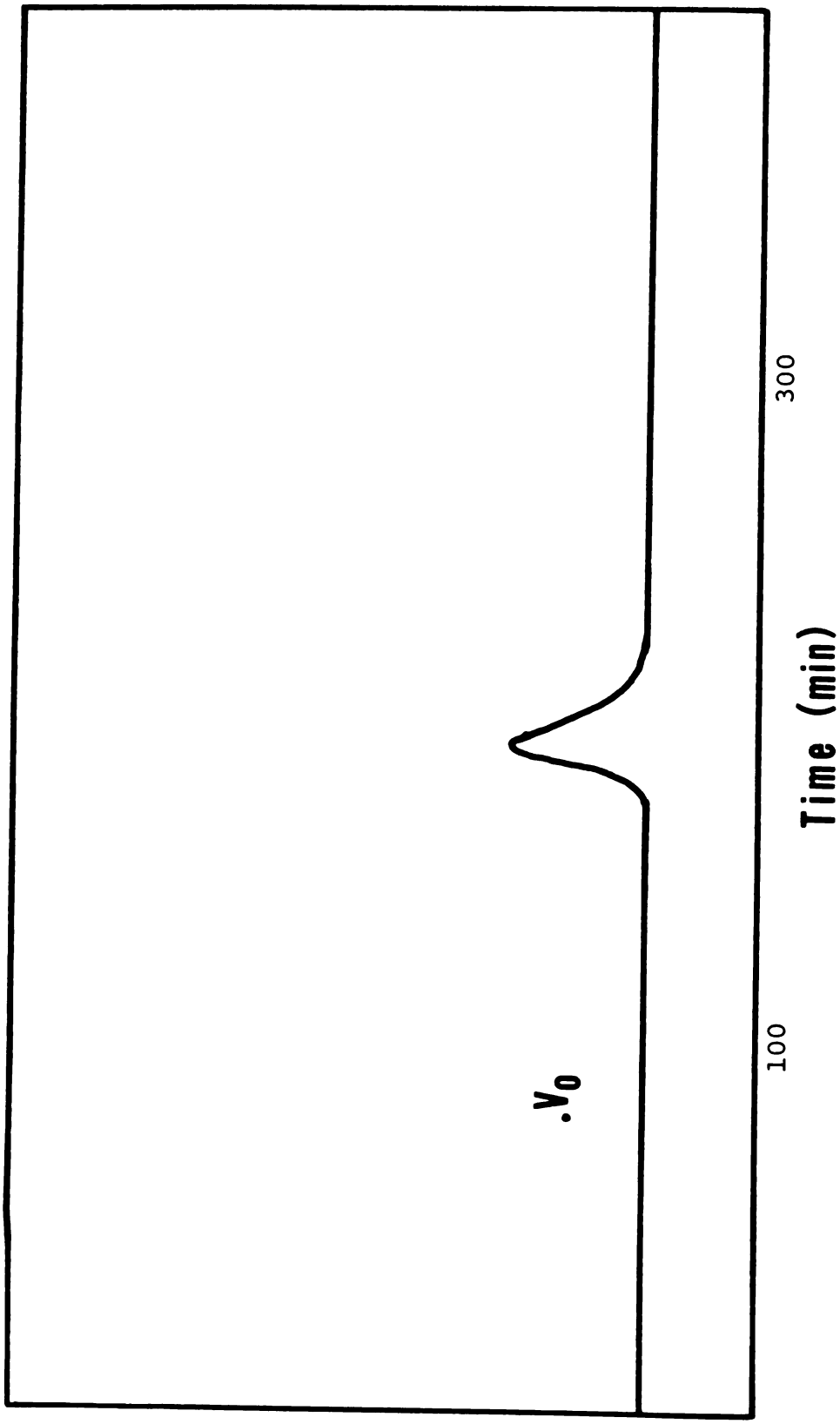

Figure 8.

The molecular weight of these peptides, therefore, appears to be greater than 700, but less than 5,000 Daltons. This observation is consistent with the findings of Kirk et al. (1972) who reported the molecular weights of the peptides to be between 1,500 and 3,600 Daltons using Bio-Rad P-2 and P-4.

Detection of Glycopeptides

Glycopeptides were distinguished on silica gel thin-layer plates by the successive application of ninhydrin and orcinol-sulfuric acid reagent, as described in the experimental methods section.

As shown in Figure 9, peptides pos 2-1 and pos 2-2 gave a brownish spot when sprayed with the orcinol-sulfuric acid reagent, indicating that these peptides may be glycopeptides. The presence of glycopeptides associated with β-Lg was quite surprising, since only $\beta\text{-Lg}_{\text{Droughtmaster}}$ had previously been reported to contain carbohydrate moieties.

Figure 9.--Thin-layer chromatographic analysis for glycopeptides. Support: silica gel G. Mobile phase: butanol-acetic acid-water (80:20:20). Left to right: plate 1 sprayed with ninhydrin reagent, plate 2 sprayed with ninhydrin reagent plus orcinol-sulfuric acid reagent.

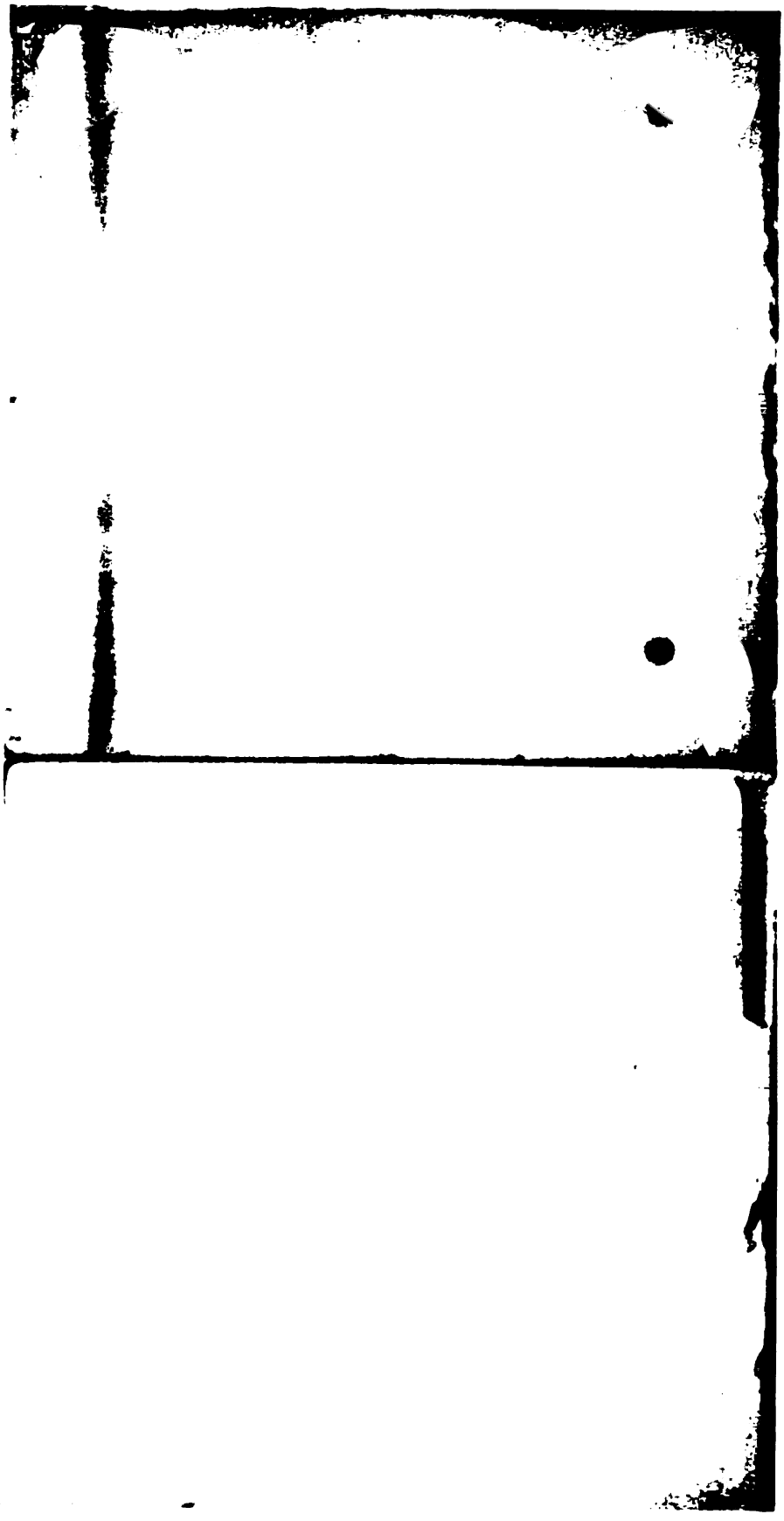

Figure 9.

Amino Acid Analysis of the Peptide Mixture

Amino acid analyses of the peptide mixture were performed on 22 hour hydrolysates of the peptide mixture (Table 2). Glycine and glutamic acid were present in high concentrations. This result is consistent with the observations of Kirk (1971) who also reported high concentrations of glutamic acid and glycine in individual peptides. It was theorized that if peptides were being released from β-Lg, then (E)β-Lg should have shown a decrease in these two amino acids. However, (E)β-Lg showed slightly higher concentrations of glycine and glutamic acid, although the difference could be explained by experimental error. Doubt was, therefore, cast as to whether these peptides were actually associated to β-Lg, or an artifact of the isolation procedure.

Nitrogen Determination in Peptides

Micro-Kjeldahl nitrogen analyses of the peptide mixture obtained by electrodialysis of β-Lg gave a value of 2.2% nitrogen. This corresponds to only 13.75% protein.

TABLE 2.--Amino acid composition of the peptide mixture.

Amino Acid Residue	g amino acid residues/100 g protein
LYS	19.51
HIS	3.72
ARG	1.01
ASP	2.78
THR	6.44
SER	2.99
GLU	32.85
PRO	.77
GLY	14.57
ALA	4.39
CYS	----
VAL	3.62
MTH	----
I-LEU	1.86
LEU	2.46
TYR	1.19
PHEN	.96
TRYP	----
TOTAL*	100.00

*Total g amino acid residues/100 g protein

This low concentration of protein indicated that there was either a high moisture content, or that a high concentration of impurities was present in the peptides. The peptides were dried in a vacuum oven at 100°C for 24 hours and again the same low nitrogen value was obtained. Therefore, the possibility of carbohydrate impurities coming from the dialysis membrane during electrodialysis was suspected.

Electrodialysis of Deionized Water

Electrodialysis was performed according to the same procedure described previously in the experimental methods section. However, electrodialysis was carried out with only deionized water instead of β-Lg inside the dialysis membrane for 48 hours. Both the electrodialysate and diffusate were pervaporated and lyophilized. Two dimensional peptide mapping, using the same conditions previously described, was run on both samples.

Surprisingly, both samples showed the same eight peptides that had previously been isolated when β-Lg was electrodialyzed (Figure 4). It, therefore, appears that

peptides previously reported to be associated to milk proteins by Gizis et al. (1965), Dorris (1968), and Kirk et al. (1972) were artifacts of the isolation procedure.

Static Dialysis Test

Cellulose dialysis membranes were treated by two methods described previously: 1. boiling in a dilute EDTA solution, and 2. according to the procedure of McPhie (1971). All membranes were thoroughly rinsed with deionized water prior to use. Deionized water was placed inside the membranes and dialyzed against deionized water at 4°C for 48 hours. The water inside and outside the membrane was then pervaporated and lyophilized. Two dimensional peptide mapping was run on the samples, and the same eight peptides that were observed in Figure 4 were present in all samples. When fresh deionized water was simply pervaporated and lyophilized, the same eight peptides were again present in peptide maps, but in a much lower concentration.

Finally, fresh deionized water was lyophilized and checked for the presence of peptides. No evidence of peptides could be found. The deionized water was, therefore, eliminated as the source of peptide contamination.

Peptides are obviously being released from cellulose membranes during static dialysis, pervaporation, and electrodialysis. Micro-Kjeldahl nitrogen determination indicated that 0.024% nitrogen was present in the dialysis membrane.

Gel Filtration Chromatography of Beta-Lactoglobulin

It has been shown that peptides were being released from dialysis tubing during static dialysis. It is, therefore, logical to assume that peptides released during normal dialysis could contaminate or associate with proteins during their isolation and purification. Gel filtration chromatography was used to determine if the peptides were associated with native β-Lg.

β-Lg was treated with 2N sodium chloride in an effort to break any electrostatic bonds which might have formed between the peptides and β-Lg. The sample was then passed over a G-25 Sephadex column. No peptide peaks were observed.

β-Lg was also treated with 7M urea to break any apolar bonds that might have formed between β-Lg and

peptide contaminants. Again, no peptide peaks were observed when the sample was passed over a G-25 Sephadex column.

Ten percent SDS was added to β-Lg to release any associated peptides. This sample was also passed over a G-25 Sephadex column. The β-Lg was eluted in the void volume and a second peak was observed. However, the presence of any of the previously observed peptides in this peak could not be established since SDS is very difficult to remove once bound to proteins, and greatly affects their electrophoretic mobilities.

It, therefore, appears that the peptides are not bound to β-Lg in quantities that can be detected with the relatively small amounts of protein that can be applied to the Sephadex column.

SUMMARY AND CONCLUSIONS

Peptides that had previously been reported to be associated to β-Lg were isolated and partially characterized. A total of eight peptides were isolated and found to have similar properties as peptides previously observed by Gizis et al. (1965), Dorris (1968), and Kirk (1971).

However, it was shown that these peptides were an artifact of the isolation procedure and were released from the dialysis tubing even though all membranes were given an extensive pretreatment prior to use. This pretreatment had been reported by McPhie (1971) to remove all impurities from cellulose dialysis tubing.

On reviewing the procedure used to manufacture cellulose tubing, it is difficult to determine where these peptide impurities were introduced. It seems most logical to assume that the peptides were actually present in the original wood pulp used as a cellulose source in the manufacture of cellulose dialysis tubing.

Peptides were shown to be released by static dialysis and pervaporation, as well as, by electrodialysis. These

peptides are, therefore, being released during normal dialysis procedures, such as the desalting of proteins. Although association of these peptides to β-Lg could not be confirmed, the possibility exists that other proteins could bind these peptides during dialysis.

Since the peptides are present in such small quantities, they should not affect the physical and chemical properties of major protein fractions being studied. However, if minor protein fraction components are being studied, these peptide artifacts could lead to reproducible but otherwise meaningless results.

In conclusion, the results of this study indicate that peptides are released from cellulose dialysis tubing, even after careful pretreatment of the tubing.

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APPENDIX

APPENDIX

Isolation of Beta-Lactoglobulin

Ten liters of fresh whole milk were collected at the Michigan State University Dairy Barn. This mixed herd milk, without being allowed to cool, was heated to 40^{0}C. Twenty grams of anhydrous sodium sulfate per hundred milliliters of milk were slowly added, stirring continuously until all the sodium sulfate was dissolved.

The temperature of this solution was then lowered to 25^{o}C and filtered through E and D No. 515 fluted filter paper. The precipitate containing the globulins, proteose peptones, casein, and fat was discarded. The filtrate containing the β-Lg, α-lactalbumin, and serum albumins was saved for further fractionation.

One milliliter of concentrated hydrochloric acid was then added per hundred milliliters of filtrate to adjust the pH to approximately 2. The resulting precipitate containing all the proteins except β-Lg was removed by centrifugation at 8,000 r.p.m. for 30 minutes. The

supernatant was decanted from the centrifuge bottles and filtered through S and S No. 478-1/2 analytical grade fluted filter paper to assure removal of the precipitated proteins.

The volume of the filtrate containing β-Lg was measured and the pH adjusted to 6.0 with concentrated ammonium hydroxide (required about .6 ml. per 100 ml. of filtrate). Twenty grams of ammonium sulfate per hundred milliliters of filtrate were then added, and resulted in the precipitation of β-Lg. After all the ammonium sulfate had been added and dissolved, the solution was permitted to sit quiescently for one hour to allow the protein to flocculate. This was followed by filtration under vacuum through a thick layer of filter aid (Johns Mansville Hyflo Super-Cel or SuperCel) deposited on Whatman #1 filter paper. The filtrate was discarded.

After filtration, the filter cake was slurred with approximately 100 ml. of deionized water and placed in a treated cellulose dialysis membrane. The contents were dialyzed overnight against deionized water and then removed from dialysis and filtered with suction through Whatman #1 filter paper in a Buchner funnel. The filter aid remaining on the filter paper was discarded.

The pH of the filtrate was adjusted to 5.8 with 1N hydrochloric acid, a few drops of toluene were added, and the solution was dialyzed against deionized water at 4°C for 48 hours (dialysate was changed every 12 hours).

Then the β-Lg solution was removed from the membrane, and the pH was reduced to 5.2 with 1N hydrochloric acid, which produced some cloudiness. A small amount of analytical grade sodium chloride was added to redissolve this precipitate. Dialysis was then continued for 12 hours, during which time β-Lg crystals formed.

Recrystallization and Purification

β-Lg crystals from several isolations were suspended in deionized water and analytical grade sodium chloride was added until all the β-Lg was dissolved. The resulting solution was then filtered through analytical grade filter paper and the pH was adjusted to 5.2 with 1N HCl. It was then placed in treated cellulose membranes with 1 ml. of toluene. The solution was dialyzed against deionized water at 4°C until β-Lg crystals formed. β-Lg was recrystallized four times using this procedure.

Nitrogen

Micro-Kjeldahl nitrogen analyses were performed using 5 to 10 ml. protein samples. The samples were heated in 4 ml. of digestion mixture over a gas flame until they were colorless. The digestion mixture consisted of 5.0 grams $CaSO_4$ $5H_2O$ and 5.0 grams SeO_2 in 500 ml. of concentrated sulfuric acid. Following this initial digestion, the mixture was cooled, 1 ml. of 30% H_2O_2 was added, and digestion was continued for the same amount of time as the first digestion. After this digestion, the flasks were again cooled, and 10 ml. of water were added to each one.

Each digestion mixture was neutralized with 25 ml. of 40% NaOH, and the released ammonia was steam distilled into 15 ml. of a 4% boric acid solution containing five drops of methyl red-bromcresol green indicator (400 mg. bromcresol green and 40 mg. methyl red dissolved in 100 ml. of 95% ethanol). Distillation was continued until the receiving flask contained a total volume of 75 ml. The ammonia was titrated with 0.0182N HCl. An internal standard was always run to compensate for any losses.

Amino Acid Analysis

Amino acid analyses were performed on 22 hour hydrolysates of β-Lg, (E)β-Lg, and the peptide mixture. Eight milligram samples were weighed directly into 10 ml. ampules. Six milliliters of 6N HCl were added to each ampule. The contents of the ampule were then frozen in a dry ice-ethanol bath, and put under a vacuum with a high vacuum pump. They were allowed to melt and the air was evacuated. The contents were then refrozen while still under vacuum, and sealed using a propane torch.

The sealed ampules were placed in a 110°C oil bath and hydrolyzed for 22 hours. The samples were then removed and cooled to room temperature.

The ampules were opened and 1 ml. of a norleucine standard was added so that transfer loss could be determined. The ampule contents were then evaporated to dryness in small round-bottom flasks on a rotary evaporator at 50°C. Each sample was dissolved in deionized water and re-evaporated until no HCl odor was detectable.

Five milliliters of 0.067M citrate-hydrochloric acid buffer, pH 2.2, were added to the dried hydrolysates.

The amino acid composition of each solution was determined using a Beckman Amino Acid Analyzer Model 120C, which utilizes a sulfonated polystyrene ion exchange resin. Aliquots of .2 ml. of each protein hydrolysate were applied to the ion exchange column for amino acid analysis.

The area under the amino acid peaks drawn by the recorder was divided by an experimentally determined standard for each respective amino acid to obtain the number of micromoles of each amino acid in the aliquot assayed. The amount of each amino acid present in the hydrolysate could then be quantitated.

The amino acid composition of the protein samples could then be expressed either as grams of amino acid per 100 grams of protein, or as moles of amino acid per 1000 moles of residue.

Polyacrylamide Gel Electrophoresis

Discontinuous polyacrylamide gel electrophoresis was performed by a modification of the procedure as described by Melachouris (1969). An E - C Vertical Gel

Electrophoretic Chamber was used for electrophoresis. The discontinuous gel system required two solutions: 1. a running gel solution, and 2. a spacer gel solution. The running gel solution (9% gel) was prepared by dissolving 45 g. Cyanogum 41 in .380M tris-hydroxymethyl-aminomethane (TRIS) - HCl buffer, pH 8.9, and was made to a volume of 500 ml. To this solution was added 0.5 ml. of N, N, N', N' - tetramethylethylenediamine (TEMED). The spacer gel solution (5% gel) was prepared by adding 25 g. of Cyanogum 41 to 0.062M Tris - HCl buffer, pH 6.7, and was made to a volume of 500 ml. To the latter solution was also added 0.5 ml. TEMED. Both the running and spacer gel solutions were stored at 4°C and brought to room temperature before use.

The E - C cell was placed on a 45° incline and 50 ml. of running gel containing 1 ml. of a 10% (w/w) ammonium persulfate solution was poured inside and allowed to polymerize. The cell was then placed in an upright position. The running gel bed was formed with 90 ml. of running gel containing 2 ml. of the 10% ammonium persulfate solution. After polymerization, the cell was placed horizontally and the remainder of the gel bed was filled with 60 ml. of spacer gel containing 1 ml. of

ammonium persulfate. Before polymerization, a slot former was placed in the spacer gel solution approximately 0.5 cm. from the interface of the running gel. Following polymerization, the E - C cell was again placed in an upright position and the buffer tanks, fitted with platinum electrodes, were filled with .046M Tris - glycine buffer at pH 8.3. The slot former was then carefully removed.

Protein samples were dissolved in the spacer gel buffer at a concentration of approximately 2% (0.02 g. protein per ml. buffer). Bromphenol blue was added as a marker dye. Sucrose was added to the sample to increase the density and insure good sample layering in the spacer gel. Approximately 10 to 25 µl. of sample was inserted in each slot. Electrophoresis was conducted at 80 ma. and continued until the bromphenol blue marker had migrated about 15 cm. from the sample slots.

Staining Polyacrylamide Gels

After completing gel electrophoresis, the polyacrylamide gels were removed from the E - C cell and

stained for 10 minutes in an Amido Black dye consisting of 250 ml. of deionized water, 250 ml. of methanol, 50 ml. of glacial acetic acid, and 5 g. of Amido Black. Excess dye was removed by soaking the gel in 7% acetic acid.