Essentials in bioresearch

ASBMB/AAI '90
Poster Presentation

The Analysis and Purification of Human Milk Whey Proteins by a Multi-Chromatographic Mode Approach

George Vella, Kevin O'Connor, Lynn Jordan and Charles Phoebe, Jr. (Sponsor: J. Li)
Waters Chromatography Division of Millipore
INTRODUCTION

Human milk proteins are of immunological, endocrinological, physiological and nutritional significance to a breast-fed neonate. Growth factors, host defense factors and digestive enzymes are some of the known properties which reside in the soluble protein fraction of human milk. Although the major proteins (alpha-lactalbumin, lactoferrin, lysozyme and the immunoglobulins) in milk whey have been investigated extensively, there are many other proteins, peptides and carbohydrates that are present in much smaller concentrations and consequently are more difficult to study unless a specific sensitive assay is developed. After the whey proteins were separated from casein by pH adjustment to 4.3, a rapid high performance liquid chromatographic reversed phase separation was developed which permitted the analysis of both the major and minor whey proteins by using conditions typically employed in peptide mapping. These conditions were chosen so that a different separation selectivity may provide the resolution of components which may not have otherwise been separated by traditional modes of chromatography used for protein purification. In addition, optical clarity at the low UV range permitted high sensitivity detection at 214nm of some of the less abundant species present in the whey fraction.

Presented in this paper, is a multi-chromatographic mode analysis of fractions collected from a large scale (21ml of whey, representing 200mg protein) reversed phase separation of human milk whey proteins. The large scale separation was carried out on three 25x100mm Prep-Pak cartridges packed with Delta-Pak C18, 15u, 300Å arranged in a stackable configuration using a 25x10 cartridge holder and two extension tubes. Gradient elution was accomplished by an increase in acetonitrile containing 0.1% TFA. Fractions collected from the preparative separation were lyophilized and resuspended in water to yield protein concentrations ranging from 0.3mg/ml to 57mg/ml. Nine of the twenty-four fractions were analysed by high performance gel filtration chromatography (GFC), anion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and high resolution reversed phase chromatography (RP) employing a different gradient elution profile. Commercially available human alpha-lactalbumin, lactoferrin and lysozyme were used as standards to aid in the orientation of the chromatographic profiles.

Much information on the physio-chemical nature of the components of human milk whey can be obtained by the multi-mode approach described. This study represents the first of a series in an attempt to identify some of the minor factors which play important roles in normal infant development. Future studies will include amino acid analysis and sequence analysis of purified components.
CHROMATOGRAPHIC CONDITIONS

REVERSE PHASE CHROMATOGRAPHY (RP)

Column: Delta-Pak™ C18, 5µ, 300Å, HPI Column (3.9 x 150mm)
Detection: 214 nm
Eluent A: Water with 0.1% TFA
Eluent B: Acetonitrile with 0.1% TFA
Gradient: 0%B to 46%B in 70 min. to 80%B in 10 min. hold 10 min.
Flow Rate: 0.5 ml/min
Column Temp: 30 °C

GEL FILTRATION CHROMATOGRAPHY (GFC)

Columns: Protein KW-802.5, 803, and 804
Detection: 214 nm
Eluent A: 50 mM Na2HPO4, pH 7.0 with 300 mM NaCl
Flow Rate: 0.5 ml/min

ANION EXCHANGE CHROMATOGRAPHY (IEX)

Column: Protein-Pak™ DEAE 8HR (10 x 100 mm)
Detection: 280 nm
Auto Blend™ Method: Tris Buffer System
Initial: 20 mM Tris, pH 8.5
Final: 20 mM Tris, pH 8.0 plus 800 mM NaCl
Gradient Duration: 35 min.
Flow Rate: 1.77 ml/min

HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

Column: Protein HIC PH-814 (8 x 75 mm)
Detection: 280 nm with baseline subtraction
Buffer A: 1.7 M (NH4)2SO4 plus 100 mM Na2HPO4, pH 7.0
Buffer B: 100 mM Na2HPO4
Gradient: 0%B to 100%B in 30 min.
Flow Rate: 1.0 ml/min
PREPARATIVE REVERSE PHASE CHROMATOGRAPHY

Column: Delta-Pak™ C18, 15µ, 300Å, Prep-Pak™ Cartridges (25 x 300mm)
Detection: 214 nm
Eluent A: Water with 0.1% TFA
Eluent B: Acetonitrile with 0.1% TFA
Gradient: 30%B to 80%B in 60 min.
Flow Rate: 19.5 ml/min
Sample: Human Milk Whey
Load: 21 ml (200 mg protein)
Crude Human Milk Whey

0507MILK2 Manual Inj 1 Ch 1

IEX

0.040
0.030
0.020
0.010
0.000
0.0

AU

Minutes

0 10 20 30 40

0518MLKSTD2 Manual Inj 1 Ch 1

HIC

0.4
0.3
0.2
0.1
0

AU

Minutes

0 10 20 30 40
Fraction 3

523WHEY04 Manual Inj 1 Ch 1

RP

GFC

mV

Minutes
Fraction 3

IEX

HIC

Minutes
Fraction 13

0523FHCT13 Manual Inj 2 Ch 1

IEX

HIC

Minutes
Fraction 14

525WHEY02 Manual Inj 1 Ch 1

mV

0 100 200 300 400

R14_209HH04 Manual Inj 1 Ch 1

mV

0 50 100 150 200

Minutes

0 20 40 60 80
Fraction 14

IEX

HIC

0523FRCT14 Manual Inj 1 Ch 1

s0523FRCT14 Vial 2 Inj 2 Ch 1

AU

Minutes
Fraction 15

530WHEY04 Manual Inj 1 Ch 1

RP

GFC

Minutes
Fraction 16

530WHEY05 Manual Inj 1 Ch 1

RP

GFC

mV

mV

Minutes

Minutes
Fraction 18

529WHEY02 Manual Inj 1 Ch 1

RIB 209HH04 Manual Inj 1 Ch 1

RP

GFC

mV

Minutes
Fraction 18

0523FACT18 Manual Inj 1 Ch 1

IEX

HIC

Minutes
Fraction 19

530WHEY01 Manual Inj 1 Ch 1

RP

GFC

Minutes
Fraction 19

Fractions 0523FRCT19 Manual Inj 1 Ch 1

**IEX**

**HIC**

Minutes 0 10 20 30 40

AU 0.010 0.008 0.006 0.004 0.002 0.000 -0.002

AU 0.010 0.008 0.006 0.004 0.002 0.000 -0.002
Fraction 24

530WHEY06 Manual Inj 1 Ch 1

R24_209HH04 Manual Inj 1 Ch 1

GFC

RP

mV

mV

0 200 400

0 600

10 20 30 40 50 60 70 80

Minutes
CONCLUSIONS

This approach demonstrates that large scale reversed phase chromatography serves as a useful first step in the separation of the various components in human milk whey. Analysis of the concentrated fractions by size exclusion, charge and hydrophobicity in the presence of high salt or organic modifiers provides much information on the physico-chemical nature of the components contained therein and forms the foundation for future studies in elucidating their identity.