DEVELOPMENT OF METHODS FOR CAPILLARY ISOELECTRIC FOCUSING OF DAIRY PROTEINS

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Abstract

Capillary Isoelectric Focusing (CIEF) is a high-resolution technique which can be applied to the separation and characterisation of complex biological mixtures such as dairy proteins. Although dairy proteins are commonly analysed by traditional gel electrophoresis techniques including 2-Dimensional PAGE, CIEF offers the advantages of reduced analysis times, the ability to handle smaller sample volumes and increased sensitivity with improved separation efficiencies.

Several methods for capillary isoelectric focusing of dairy proteins have been developed herein. For the analysis of soluble whey proteins methods that can be used with either UV or mass spectrometry (MS) detection have been set up. For MS detection a coaxial sheath flow interface in conjunction with electrospray ionisation has been utilised. For analysis of the inherently insoluble casein proteins with UV detection denaturing and reducing agents have been introduced into the system. Results have shown very close similarities to those obtained by IEF gels.

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List of Abbreviations

2D	Two Dimensional
α-csn	α-Casein
α-Lac	α-Lactalbumin
Amy	Amyloglucosidase
β-csn	β-Casein
β-Lac	β-Lactoglobulin
β-Lac-A	β-Lactoglobulin-A
β-Lac-B	β-Lactoglobulin-B
BME	β-Mercaptoethanol
BSA	Bovine Serum Albumin
CA	Carbonic Anhydrase II
CCK	CCK Flanking Peptide
CE	Capillary Electrophoresis
CEC	Capillary Electrochromatography
CGE	Capillary Gel Electrophoresis
CIEF	Capillary Isoelectric Focusing
CITP	Capillary Isotachophoresis
CZE	Capillary Zone Electrophoresis
DNA	Deoxyribosenucleic Acid
DTT	$_{DL}$-Dithiothreitol
EDTA	Ethylenediaminetetra-Acetic Acid
EOF	Electroosmotic Flow
ESI	Electrospray Ionisation
GMP	Glycomacropeptide
HPLC	High Performance Liquid Chromatography

i.d.	Internal Diameter
IEF	Isoelectric Focusing
Ig	Immunoglobulin
IgG	Immunoglobulin G
κ-csn	κ-Casein
kV	Kilo Volt
Lf	Lactoferrin
Lp	Lactoperoxidase
mA	Milli Amps
Mb	Myoglobin
Mb-A	Myoglobin Acidic
Mb-B	Myoglobin Basic
MEKC	Micellar Electrokinetic Chromatography
MFGM	Milk Fat Globule Membrane
mg	Milli Gram
MHEC	Methyl 2-hydroxyethyl cellulose
mL	Milli Litre
MOPS	3-[N-Morpholino]propane-sulfonic acid
MS	Mass Spectrometery
MWCO	Molecular Weight Cut Off
NaOH	Sodium Hydroxide
nL	Nano Litre
PAGE	Polyacrylamide Gel Electrophoresis
PDA	Photo Diode Array
pI	Isoelectric Point (of a protein or peptide)
PP5	Proteose Peptone 5
PSI	Pounds per Square Inch

Rb	Ribonuclease
RNA	Ribosenucleic Acid
RP	Reversed Phase
SDS	Sodium Dodecylsulfate
TCA	Trichloroacetic Acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TI	Trypsin Inhibitor
TIC	Total Ion Count
Tris	Tris(hydroxymethyl)-aminomethane
Tryp	Trypsinogen
µg	Micro Gram
µL	Micro Litre
UV	Ultraviolet
V/cm	Volts per Centimetre (of column length)
v/v	Volume to Volume
v/w	Volume to Weight

1 Overview

Capillary Isoelectric Focusing (CIEF) is a technology that has developed in the last few years and is a technique whereby proteins and peptides are separated according to their isoelectric point (*pI*); such separations are generally as good as those obtained by flat bed isoelectric focusing (IEF) polyacrylamide gel electrophoresis (PAGE). Advancements in CIEF technology have been led by the requirements of proteomic research for high throughput analysis coupled with limited sample size. Routine methods for CIEF involve ultraviolet (UV) detection, but mass spectrometry (MS) detection is becoming more popular for many research groups. This is analogous to the time consuming method of 2-dimensional IEF/ PAGE in which spots on gels are excised, digested with enzyme, and the digests analyzed by high performance liquid chromatography-MS (HPLC-MS). CIEF-MS has the capability to reduce analysis times considerably and is used for a number of applications. Detection is of intact protein rather than hydrolyzed protein, which saves time on database searches. In recent years the CIEF-UV method that has traditionally only had applications to water soluble protein, has been modified for separation of proteins in denaturing systems. In this way proteins that are inherently insoluble can be separated by CIEF. Currently there is only one CIEF method within the literature that has a dairy application and this is based on the monitoring of glycosylation products of glycomacropeptide (GMP) (Tran et al. 2001).

Over the last few years dairy industries around the world have embarked on large-scale proteomic research, with a view to one or more of the following:

a.) The discovery of low abundance proteins and peptides that may have potential health benefit that could be explored in niche products of the future.

b.) Understanding expression and co-regulation of milk proteins.

c.) Acquisition of intellectual property for future strategic use.

The competitive edge of a dairy company is governed partly by the speed in which fundamental research can be translated into a commercial process or product. In this

respect it is mandatory to identify new technological areas and analytical techniques that may allow large time and cost savings in the commercialization pipeline. Capillary electrophoresis (CE) is one such analytical tool as it is rapid, has very good detection limits, can be interfaced to MS detection and requires very small sample size.

The aim of this research was to develop new methods in CE analysis that would be applicable to a wide variety of dairy-based samples, and could be used as rapid screening methods for proteomic applications. The CE mode of CIEF was investigated, as sample size in this format is generally 20 times larger than other modes of CE, thus enhancing detection sensitivity, and the method is able to separate proteins and peptides over a wide range of *pI* values. The method has the additional advantage that *pI* values can help in the identification of unknown protein. The technique is also very rapid and gives very good comparison to the IEF gel format, making this technology very much cheaper and less labour intensive to use.

Bovine dairy proteins are comprised of two main groups, the casein and the whey proteins. Caseins make up approximately 80 % of dairy protein and typically occur as micelles in milk, being inherently insoluble. Whey proteins on the other hand make up the remaining 20 % of protein and tend to be globular water-soluble proteins, while in addition there is another group of proteins collectively termed the milk fat globule membrane (MFGM) protein that makes up a very small amount (<1 %) of protein in milk. Taking these general properties into consideration the overall aim of this thesis was to develop methods of CIEF for the different types of dairy protein as follows:

- Develop methods using UV detection that are simple to run with minimum preparation and optimized for:
    - The major whey proteins
    - Casein proteins
    - Fractionated protein samples
- Compare these methods to IEF flat bed PAGE
- Develop methods of CIEF-MS for soluble proteins and if possible modify the method for insoluble proteins
- Compare CIEF-MS results to two dimensional PAGE (2D-PAGE) methods
- Compare CIEF methods to already developed CZE methods where applicable