WHEY PROTEIN FRACTIONATION BASED ON SP-SEPHAROSE CATION EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Bovine whey protein consists of different type of proteins such as β-lactoglobulin (β-lag), α-lactalbumin (α-lac), immunoglobulin, bovine serum albumin (BSA), lactoferrin, lactoperoxidase and glycomacropeptide. Single protein is more valuable compare to the mixture of proteins and it can be used for specific application. The main objective of this study is to fractionate whey protein components into several groups or single protein fraction using SP-sepharose^{TM} Fast Flow (Amersham Biosciences) cation exchange chromatography column. The chromatographic process was run at different pH range from pH 4 to pH 8 using 24 ml column. All major whey proteins were recovered in the elution fraction at pH 4 and pH 4.5. No protein was bound at pH 7 and pH 8. The best whey fractionation from 2 ml whey feed was achieved at pH 4 with the yield for β-lag, BSA and α-lac are 83.33%, 66.44% and 10.51% respectively. Different protein fraction recovered during fractionation process at different pH using cation exchange chromatography process, can be used as a guideline to isolate particular protein of interest from bovine whey.

ABSTRAK

Whey protein daripada susu lembu terdiri daripada beberapa jenis protein seperti β-lactoglobulin (β-lag), α-lactalbumin (α-lac), immunoglobulin, bovine serum albumin (BSA), lactoferrin, lactoperoxidase dan glycomacropeptide. Protein tunggal adalah lebih bernilai jika dibandingkan dengan campuran protein dan ia boleh digunakan untuk aplikasi tertentu. Objektif utama kajian ini dijalankan adalah untuk mengasingkan komponen whey protein kepada beberapa kumpulan atau protein tunggal dengan menggunakan SP-sepharose^{TM} Fast Flow (Amersham Biosciences) cation exchange chromatography column. Proses kromatografi dijalankan pada pH berbeza antara pH 4 hingga pH 8 menggunakan kolum bersaiz 24 ml. Kesemua protein utama whey di perolehi di dalam pecahan elusi pada pH 4 dan pH 4.5. Tiada protein yang terikat pada kromatografi kolum pada pH 7 dan pH 8. Pengasingan terbaik dari 2 ml whey di capai pada pH 4 dimana peratusan yield masing-masing untuk β-lag, BSA dan α-lac adalah 88.33%, 66.44% dan 10.51%. Pecahan protein yang berlainan komposisi yang di perolehi dari proses pengasingan pada pH yang berlainan menggunakan proses cation exchange kromatografi boleh di gunakan sebagai panduan untuk mendapatkan protein yang tertentu daripada whey protein.

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LIST OF ABBREVIATIONS

α-lac	-	α-lactalbumin
β-lag	-	β-lactoglobulin
Ag^+	-	Silver
BSA	-	Bovine Serum Albumin
CH_3COO^-	-	Acetate
Cl^-	-	Chloride
CO_2	-	Carbon dioxide
DEAE	-	Diethylaminoethyl cellulose
F^-	-	Fluoride
H^+	-	Hydrogen
HCl	-	Hydrochloric acid
$HCOO^-$	-	Formate ion
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	-	High Performance Liquid Chromatography
I^-	-	Iodine
Ig	-	Immunoglobulins
IgA	-	Immunoglobulin A
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
K^+	-	Potassium
KH_2PO_4	-	Potassium Phosphate, mono-sodium salt
K_2HPO_4	-	Potassium Phosphate, di-sodium salt
L	-	Litre
Li^+	-	Lithium
M	-	Molarity
MeCN	-	Acetonitrile
MES	-	2-(N-morpholino)ethanesulfonic acid
ml	-	Millilitre
MOPS	-	3-(N-morpholino)propanesulfonic acid
Na^+	-	Sodium
NaCl	-	Sodium Chloride
NaH_2PO_4	-	Sodium Phosphate, mono-sodium salt
NaOH	-	Sodium Hydroxide
Na_2HPO_4	-	Sodium Phosphate, di-sodium salt
NH_4^+	-	Ammonium
NO_3^-	-	Nitrate
OH^-	-	Hydroxide
pI	-	Isoelectric Point
PIPES	-	Piperazine-N,N′-bis(2-ethanesulfonic acid)

PO_4^{3-}	-	Phosphate
RPC	-	Reverse Phase Chromatography
SDS-PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SP	-	Sulphopropyl
TFA	-	Trifluoroacetic acid
Tris	-	Tris(hydroxymethyl)aminomethane
UV	-	Ultraviolet

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Caseins and whey proteins are two major proteins present in the bovine milk. In whey proteins, different types of proteins exist such as β-lactoglobulin (β-lag), α-lactalbumin (α-lac), immunoglobulins (Ig), bovine serum albumin (BSA), lactoferrin, lactoperoxidase, glycomacropeptide. These whey proteins have very high pharmaceutical value (Hahn et al., 1996). However, the separation process involves in fractionation them into individual protein is very challenging.

There are several techniques or methods commonly used to separate whey proteins component such as by using chromatography or membrane based separation and also precipitation techniques. Chromatography technique, particularly ion-exchange adsorption process, has been developed and utilized successfully on a commercial scale for whey proteins separation and whey protein concentrates production (Gerberding and Byers, 1998).

Based on the fact that single proteins component can give more benefit compare to its present in the mixture form, this study was conducted to fractionate the whey protein component by using SP sepharose cation exchange chromatography.

1.2 Problem Statements

Single protein isolation from bovine whey protein is very challenging task. Some components of whey protein have very similar properties and exist at various

levels of concentrations. By exploiting the different in protein isoelectric point (pI), SP sepharose cation exchange chromatography was used in this study to fractionate whey protein into several fraction or single protein component. This defined fraction or pure protein fraction can further be used for specific application and will have a better value compared to its original composition.

1.3 Objective of Study

The main objective of this study is to fractionate whey protein components into several groups of protein or single protein fraction using SP sepharose cation exchange chromatography by optimizing the operation pH during the purification process.

1.4 Scopes of Study

In order to fulfill the research objective, the following scopes were outlined:

1. Preparation of whey protein solution from bovine milk.
2. Setup and operated cation exchanger chromatography using AKTA Explorer 100 liquid chromatography system.
3. Study the fractionation of bovine whey protein at different pH from pH 4 to pH 8.
4. Analysis of protein fraction by using reverse phase chromatography techniques.

CHAPTER 2

LITERATURE REVIEW

2.1 Composition of Bovine Milk

Bovine milk consists of water, proteins, carbohydrates, lipids, vitamins, minerals and growth factors. Bovine milk generally contains 30-35 g L^{-1} protein and is commonly divided into two classes based on the solubility at pH 4.6 which is the insoluble caseins that represent approximately 80% of total milk protein, and the soluble whey proteins that represent approximately 20% of total milk protein (Robinson, 2002, Walstra et al., 2006). Both the casein and whey protein groups are heterogeneous (Robinson, 2002). Bovine whey proteins consist of different types of proteins such as β-lactoglobulin (β-lag), α-lactalbumin (α-lac), immunoglobulins (Ig), bovine serum albumin (BSA), lactoferrin, lactoperoxidase, and glycomacropeptide. Table 2.1 shows the concentrations of different proteins in milk and Table 2.2 shows the protein composition and properties of bovine whey according to Hahn et al. (1998).

2.1.1 β-Lactoglobulin

β-Lag is the most abundant whey protein and represents about 50% of the total whey protein in bovine milk. There are eight known genetic variants of β-Lag: A, B, C, D, E, F, G and Dr. The A and B genetic variants are the most common and exist at almost the same frequency. β-Lag has a molecular weight of 18 kDa and contains two internal disulfide bonds and a single free thiol group, which is of great importance for changes occurring in milk during heating (Robinson, 2002).

Table 2.1: Typical concentration of proteins in bovine milk (Robinson, 2002)

	Grams/Liter	% of total protein
Total protein	33	100
Total caseins	26	79.5
α_{s1}-Casein	10	30.6
α_{s2}-Casein	2.6	8.0
β-Casein	9.3	28.4
κ-Casein	3.3	10.1
Total whey proteins	6.3	19.3
α-Lactalbumin	1.2	3.7
β-Lactoglobulin	3.2	9.8
BSA	0.4	1.2
Immunoglobulins	0.7	2.1
Proteose peptone	0.8	2.4

Table 2.2: Protein composition of bovine whey according to Hahn et al. (1998).

Protein	Average concentration in whey (g/L)	Molecular mass ($\times 10^{-3}$)	Isoelectric point, pI
α-lactalbumin (α-lac)	1.5	14.2	4.7-5.1
Bovine Serum Albumin (BSA)	0.3-0.6	69	4.9
β-lactoglobulin (β-lag)	3-4	18.4	5.2
Immunoglobulins (Ig)	0.6-0.9	150-900	5.8-7.3
Lactoferrin	0.05	78	8.0
Lactoperoxidase	0.06	78	9.6

2.1.2 α-Lactalbumin

α-Lac accounts for about 20% of the whey proteins and has three known genetic variants. It has a molecular weight of 14 kDa and contains four interchain disulfide bonds. α-Lac binds two atoms of calcium very strongly, and it is rendered susceptible to denaturation when these atoms are removed (Robinson, 2002).

2.1.3 Bovine Serum Albumin

BSA represents about 5% of the total whey proteins and is identical to the serum albumin found in the blood. The protein is synthesized in the liver and gains entrance to milk through the secretory cells. It has one free thiol and disulfide linkages, which hold the protein in multiloop structure (Robinson, 2002). Serum albumin appears to function as a carrier of small molecules, such as fatty acids, but any specific role that it may play is unknown (Robinson, 2002).

2.1.4 Immunoglobulins

Igs which are approximately 10% of the whey protein are antibodies synthesized in response to stimulation by macromolecular antigens foreign to the animal. They also are polymers of two kinds of polypeptide chains which is light (L) of molecular weight 22.4 kDa and heavy (H) of molecular weight 50-60 kDa. Four types of Ig have been found in bovine milk are IgM, IgA, IgE, and IgG (Robinson, 2002).

2.1.5 Other Proteins

Several other proteins such as β-microglobulin, lactoperoxidase, lactoferrin, and transferring, both of which are iron-binding proteins, proteose peptones, and a group of acyl glycoproteins are found in small quantities in whey (Robinson, 2002).

2.2 Application of Bovine Whey Protein

Single proteins extracted from whey protein can give benefits to human and also animal nutrition. Besides that, it has a high value in pharmaceutical industries. Oral administration of bovine IgG is known to be an effective treatment of various infections for new-born infants whereas lactoferrin and lactoperoxidase are known to act as antimicrobial factors (Hahn et al., 1998).

2.3 Protein Separation Techniques

In protein separation, a variety of technique can be use to isolate a single protein of interest from a complex mixture. Protein separation is vital for the characterization of the function, interactions and structure of the interested protein. Protein separation is typically the most laborious aspect in bioproduct manufacturing. By exploiting the difference in size, physico-chemical properties and binding affinity of particular protein, precise protein separation can be achieved (Ahmed, 2005). Two most common technique used for protein separation is chromatography and membrane filtration. Table 2.3 shows several techniques for protein purification.

Table 2.3: Several techniques for protein purification (Ahmed, 2005)

Technique	Property required	Remarks	Recommended application
Membrane filtration	Molecular size	Fractionation as well as concentration. Loss of protein by non-specific adsorption.	At the beginning of a purification procedure. Particularly useful for concentrating large volumes of culture medium.
Centrifugation	Molecular size, shape, density	Commonly used for cellular fractionation.	
Preparative isoelectric focusing	pI	Proteins precipitate in the rotofor chamber	
Size exclusion chromatography	Molecular size	Usually low resolution. Provide information about protein molecular weight.	At the end of a purification procedure
Ion exchange chromatography	Charge	Protein binding capacity usually high	At the beginning of a purification procedure
Reversed phase chromatography	Hydrophobicity	Resolution varies according to gel size. Commonly used for peptide separation.	Used for separation of peptides, digested purified proteins, and other applications where loss of protein's biological activity is not a concern.
Hydrophobic interaction chromatography	Hydrophobicity		After ammonium sulfate fractionation, but before ion-exchange chromatography
Affinity chromatography	Binding ligand	Usually specific separation. Limited by availability of immobilized ligand. Expensive to scale up.	At the beginning of a purification procedure
Chromatofocusing	Charge, pI		Useful to separate isoforms of closely spaced pIs. Use after affinity chromatography

2.3.1 Chromatography

Chromatography is operated based on the partitioning of a sample between a moving phase and a stationary phase. Nowadays, chromatography is recognized as the most powerful separation method with regard to resolution and versatility, having superior resolving power and capable of isolating larger quantities of protein. Different types of chromatographic techniques have evolved, including paper, thin layer, and liquid chromatography (Rosenberg, 2004).

Chromatography can be performed using different type of interactions such as gel filtration, ion exchange, hydrophobic interaction, and affinity chromatography.
Gel filtration chromatography is also known as molecular sieving, gel permeation and size exclusion chromatography (SEC). The objective of gel filtration is to achieve rapid separation of molecules based on size. Gel filtration chromatography does not depend on the adsorption of protein to a solid phase (Wheelwright, 1991).

Hydrophobic interaction chromatography is separations based on the attraction between hydrophobic groups on the protein and a hydrophobic matrix. The sample is applied under conditions of high salt concentration and eluted under conditions of low salt concentration. Hydrophobic interaction chromatography is applicable to most proteins although the degree of separation is lower than a comparable ion exchange or affinity chromatography operation (Wheelwright, 1991).

Affinity chromatography relies on a specific interaction between the product protein and the solid phase to effect separation from contaminants in the feed. Affinity reactions allow hundred to thousand fold purifications within a single step rather than relying on relatively small differences between the product and contaminants that lead to purifications of only few fold improvements. Affinity chromatography is a concentrating technique which handling large volumes of dilute media and delivering a small volume of concentrated product (Wheelwright, 1991).

2.3.2 Membrane Separation

Membrane can be described as an interphase usually heterogenous, acting as a barrier to the flow of molecular and ionic species present in the liquids and/or vapors contacting the two surfaces. Separations with membrane do not required additives, and they can be performed isothermally at low temperatures with less energy consumption compared to other thermal separation processes. Membrane separation is mainly based on molecular size but also to a lesser extent on shape and charge.

Microfiltration (MF) and ultrafiltration (UF) are two types of membrane process that are widely used in large-scale protein purification (Wheelwright, 1991). MF and UF are differing by the size of the particles they treat. For MF, the range of the particles is from around 0.05 μm to around 2 μm. For UF, the range of the particles to be treated is from around 0.2 μm to around 200 nm (Wheelwright, 1991).

MF is commonly used to remove suspended particles from a process fluid and comprises operations such as the recovery of cells from fermentation broth and the clarification of lysed-cell slurries. UF on the other hand is an effective technique for concentrating or separating dissolved molecules of different sizes. In biotechnology, UF may be used during the initial separation of the cells from the fermentation or culture medium. It may also be used to separate the cellular fragments from the medium after the cells are broken (Wheelwright, 1991).

2.3.3 Other Techniques

Besides chromatography and membrane separation method, there are other techniques that commonly used for protein separation are precipitation, extraction, centrifugation, electrophoresis and expanded bed adsorption.

In electrophoretic process, the protein molecules are separated in an electric field at constant pH and current. Two types of electrophoretic methods are isoelectric focusing and isotachophoresis. In isoelectric focusing, the separation occurs by the pH

gradient, while in isotachophoresis, the components being separated according to the conductivities differrence.

Precipitation occurs when the solubility of the protein in solution is reduced beyond some critical value, or with addition of organic solvents. On the other hand, extraction is a process in which the protein of interest is transferred from the existing aqueous phase to another phase, either aqueous or organic.

Centrifugation is a technique used to separate particles from a solution. In the biological sciences the particles are usually cells, subcellular organelles, large molecules, or aggregates. There are two types of centrifugation procedures which is preparative that is used to isolate specific particles, and analytical which involves measuring physical properties of a sedimenting particle.

2.4 Ion Exchange Chromatography for Protein Fractionation

Ion exchange chromatography is a widely used for protein purification due to its easy to use and scale-up capabilities. The characteristic of a protein which has net positive charge in low pH and negative charge in a high pH buffer will be manipulated in this technique. Isoelectric point (pI) is a point in which the net charge of the protein is zero. Anion protein is negatively charged and has pH values above pI. Cation protein is positively charged and has pH values below pI. In ion exchange separations, the distribution and net charge on the protein's surface determines the interaction of the protein with the charged groups on the surface of the chromatography packing material (Rosenberg, 2004).

The chromatography media, which had covalently attached with positive functional groups, is referred to an anion exchanger and the one that attached with negative groups is called as cation exchanger. The charges on the protein and the packing material must be opposite for the exchange interaction to occur. Proteins that interact weakly with the ion exchanger will be retained on the column resulting in short retention times. Meanwhile, proteins that strongly interact with the ion exchanger will be retained and have longer retention times (Rosenberg, 2004). Figure 2.1 shows a

guideline to choose cation or anion exchange column based on the operation pH and pI of the target protein.

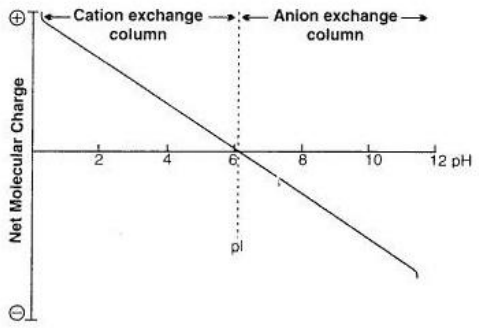

Figure 2.1 Guideline for choosing a cation or anion exchange column based on the pI of the target protein, taken from Rosenberg (2004).

Physical properties such as mechanical strength and flow characteristics, behavior towards biological substances and capacity is important in selecting the chromatographic media. Polystyrene, cellulose and polymers of acrylamide and dextran are basically three major groups of materials used in the construction of ion exchangers. The advantages of acrylamide and dextran polymers are having molecular sieving properties, which make it able to be separate on the basis of size and charge (Rosenberg, 2004).

2.5 Mechanism of Ion Exchange Chromatography

Proteins carry both positively and negatively charged groups and are called "amphoteric". The charge they carry is dependent upon the pH, with the pH value at which they have zero net charge termed the isoelectric point, pI. To determine the optimal conditions for operating an ion exchange separation, the variation in charge of the protein with pH and the stability of protein across the pH range is important (Wheelwright, 1991).

At a pH below the pI of protein, the net charge of the protein is positive, and the protein will bind to a cation exchanger. Meanwhile at a pH above the pI the net charge

will become a negative, and the protein will bind to an anion exchanger. Table 2.4 describes the conditions under which a protein does or does not adsorb to the ion exchanger matrix (Wheelwright, 1991).

Table 2.4: Conditions under which a protein binds or does not bind to the ion exchange matrix (Wheelwright, 1991).

	Cation exchanger	**Anion exchanger**	**Protein net charge**
Above pI	Does not bind	Binds	Negative
Below pI	Binds	Does not bind	Positive

Although the net charge of a molecule may be zero at the pI point, the local charge distribution is not uniform, and areas of positive or negative charge will be found across the surface of the protein. Therefore, pI alone will not always predict the point at which the molecule no longer has an affinity for the matrix although it may serve as a guide for selecting conditions for ion exchange (Wheelwright, 1991).

Proteins are labile molecules and easily denatured by high temperature, extreme pH, organic solvents, and oxidative atmosphere. It is therefore of paramount importance that during any chromatographic run these factors be taken into account. If the protein is an enzyme, simple activity assays can be conducted to determine optimal conditions for the stability of the protein. It is often a good idea to add reducing agents such as β-mercaptoethanol or dithiothreitol to prevent oxidation. Speed is also essential in chromatographic runs. The longer the process the more the protein is exposed to the oxidative atmosphere. (Rajni and Mattiasson, 2003)

Proteins are made up of amino acids that contain various chemical groups attached to a peptide backbone. These groups may be positively or negatively charged, or they may be electrically neutral. The ion exchange resin (solid support matrix) contains electrically charged species, such as carboxyl or quaternary ammonium groups, covalently attached. Associated with each linked charge is a counterion of opposite charge, bound only by ionic attraction (Wheelwright, 1991).

Charged regions of the protein containing a charge of opposite polarity to the resin will be attracted to the solid phase. The charged site on the protein will displace the counterion, and become bound to the resin by ionic interaction. Noncharged species will not become attached to the resin and may be separated by washing. The protein bound by ionic interaction can be removed or eluted from the resin by displacement with an ion having a stronger affinity for the solid phase than the protein does. (Wheelwright, 1991). Figure 2.2 illustrates the basic idea principle of ion exchange chromatography.

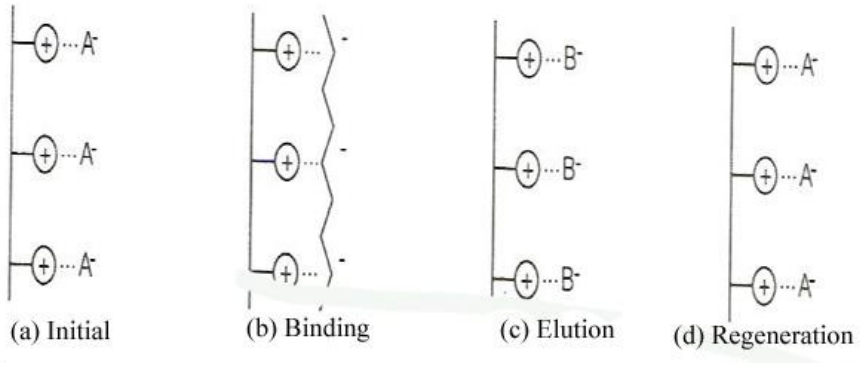

Figure 2.2 Schematic diagram illustrating mechanism of ion exchange (Wheelwright, 1991). (a) Anion exchange resin with negatively charged counterions. (b) Negatively charged protein associated with resin. (c) Elution of protein from resin. (d) Regeneration of resin to original counter ion.

2.5.1 Protein Elution Strategies

Electrostatic forces bind a protein reversibly to an ion exchange column. Raising the counter-ion (salt) concentration is the most frequently used strategy for disrupting the electrostatic force between protein and ion exchanger. At low concentrations, counter-ions (such as Na^+ or Cl^-) which are low molecular weight ions, can be dissociated from an ion exchanger by a protein and at elevated concentrations, can effectively compete with that protein for binding to the ion exchanger. A salt elution is simple to perform and is easily reproducible. Two methods exist for performing a salt elution. In a step elution, the salt concentration is increased in distinct

steps. A gradient elution utilizes a gradient maker to establish a smooth (continuous) increase in salt concentration (Bollag et al., 1996).

Besides using a positive gradient of ionic strength, bound proteins can also be eluted from the column by varying the pH of the eluent. Elution of proteins by the gradient of pH (continuous or stepwise) is not frequently employed, since some proteins may not be stable or precipitate at some pHs. Moreover, in conventional ion-exchange chromatography, a continuous pH gradient is not easy to produce at constant ionic strength and cannot be achieved by mixing buffers of different pH in linear volume ratios, since simultaneous changes in ionic strength occur (Ahmed, 2005).

2.6 Advantages of Ion Exchange Chromatography

Most biochemical have unique three-dimensional structures and charge distributions. Therefore, they can bind with ion-exchange sorbents with distinctly different affinity. As an example, replacement of two charged amino acid residues in a protein β-lactoglobulin can result in a different retention time in ion-exchange chromatography. In addition to its high selectivity, ion-exchange chromatography does not require potentially denaturing or toxic solvents (Asenjo, 1990).

The salts that are introduced in ion-exchange purification are relatively harmless. If necessary, they can be removed with a subsequent size-exclusion chromatography step. Compared to affinity sorbents, ion-exchange sorbents have higher capacities, can last longer, and are less expensive. Hence, ion-exchange chromatography has been a popular large-scale (column diameter greater than 2 cm) separation technique for the purification of amino acids, peptides, enzymes, nucleic acids, pharmaceuticals, and food products (Asenjo, 1990).

2.7 Preparation of Buffers

A stable pH of the protein environment is important since proteins are extremely heterogeneous biological macromolecules and their properties can be severely affected by small changes in hydrogen ion concentration (Ahmed, 2005).