Imunoaffinity Chromatographic Purification of Bovine Plasma Factor XIII Using Chicken’s Egg Yolk Immunoglobulin (IgY)

by

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THE UNIVERSITY OF BRITISH COLUMBIA

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Department of Food Science

The University of British Columbia
Vancouver, Canada

Date Oct 14, 1999
ABSTRACT

Bovine plasma FXIII belongs to the family of enzymes referred to as transglutaminase (EC 2.3.2.13). A novel method for purifying bovine plasma FXIII was developed, which involved the use of bovine plasma FXIII-specific egg yolk immunoglobulin (IgY) in immunoaffinity chromatography. To improve the specificity of crude IgY polyclonal antibodies against the enzyme, the antibody preparation was purified by the adsorption of specific IgY using purified bovine plasma FXIII, with or without an additional step for the removal of bovine serum albumin- (BSA-) cross-reactive antibodies using BSA as the antigen. Immunosorbents were prepared using specific IgY thus produced. It was found that a higher degree of purification of bovine plasma FXIII was attained (specific activity and purification factor of up to 316 nmol monodansylcadaverine incorporated/ 30 min/ mg protein and 64.8-fold respectively) when both purification steps with respect to specific IgY were employed. With further optimization, immunoaffinity chromatographic method using specific IgY such as that described in this study for the purification of bovine plasma FXIII should be a viable and attractive alternative to other methods currently available.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>EF</td>
<td>Enhancement Factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FC</td>
<td>“Immunosorbent with immobilized bovine plasma FXIII”</td>
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<tr>
<td>FXIII</td>
<td>Factor XIII</td>
</tr>
<tr>
<td>HFSN</td>
<td>“Heat-treated Fibrimex Supernatant” (FibriMex heated at 56°C for 4 min)</td>
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<tr>
<td>Io</td>
<td>Fluorescence intensity of control</td>
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<tr>
<td>If</td>
<td>Fluorescence intensity of sample (or unknown)</td>
</tr>
<tr>
<td>IAC-I</td>
<td>Immunoaffinity column prepared using SIGY1</td>
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<tr>
<td>IAC-II</td>
<td>Immunoaffinity column constructed using SIGY2</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IgY</td>
<td>Chicken egg yolk immunoglobulin</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgG-HC</td>
<td>Immunoglobulin G (heavy-chain)</td>
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<tr>
<td>IgG-LC</td>
<td>Immunoglobulin G (light-chain)</td>
</tr>
<tr>
<td>MDC</td>
<td>Monodansylcadaverine</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate (Na(^+)) buffer</td>
</tr>
<tr>
<td>LEE</td>
<td>“Lee’s Enzyme Extract” (Partially purified bovine plasma FXIII used for immunization of hens)</td>
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<tr>
<td>PGUB</td>
<td>“Protein G Unbound” (Bovine plasma FXIII preparations enriched by the fractionation of contaminating Ig)</td>
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<tr>
<td>RuBisCO</td>
<td>Rubilose bisphosphate carboxylase</td>
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<td>Bovine plasma FXIII-specific IgY obtained in two-step purification, first on immobilized BSA column then on FC</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>kilodaltons</td>
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<td>M</td>
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**Aunt Millie** Really miss your cooking…and thank you for caring.

**Mom and Dad** Thank you so much for your concern, support and patience. You are the greatest!

**Nachi Lau and Philip Fong** Let’s do coffee! Thank you for accepting me just as I am.
1.1 Introduction

Transglutaminase (EC 2.3.2.13) is a family of enzymes whose most studied function is its ability to catalyze the crosslinking of proteins, such as those found in food. Potential applications of these enzymes in the food industry include modification of protein functionality such as gelling capability (De Backer-Royer et al., 1992b) and emulsifying activity (Larré et al., 1993a), development of novel foods such as restructured meat (Nielsen et al., 1995) and improvement of nutritive value of proteins, among many others.

Most studies related to food technology on transglutaminase found in the literature have employed transglutaminase isolated from guinea pig liver and a microbial source (Streptoverticillium mobaraense var.). Whereas the former is undesirable for large-scale commercial applications, mostly due to high cost and scarcity, the latter has become the most important source of transglutaminase. Blood from slaughtered cows constitutes an abundant waste product in the meat industry and the recovery of bovine plasma FXIII (a transglutaminase occurs in cow's blood) could reduce pollution as well as generate added value for operators.

Bovine plasma FXIII was not commercially available during the time of this study. Several methods for the purification of bovine and other plasma FXIII could be found in the literature. The majority of these methods involved tedious procedures of multiple ammonium sulfate precipitations and chromatography. Ammonium sulphate
precipitation followed by ion-exchange chromatography of crude concentrates on DEAE-cellulose either by continuous (Lorand, 1961) or stepwise (Loewy et al., 1961) gradient elution were among the earlier methods proposed. In particular, Lorand et al. (1968) stated that they found a linear NaCl gradient to 0.2M (in 0.05M Tris-HCl buffer, pH 7.5) most satisfactory. Though the pattern and size of inactive peaks varied somewhat from preparation to preparation, elution of the activity itself at around 0.05M NaCl seemed to be very reproducible.

McDonagh et al. (1976) developed a method for affinity chromatographic purification of plasma and platelet FXIII, which was based on known structural characteristics of these molecules: Plasma FXIII is composed of a and b subunits which are held together by noncovalent interactions. The a subunits contain free sulfhydryl groups, while in the b subunits all the cysteines form disulfide bonds. An affinity gel consisting of organomercurial agarose with p-chloromercuribenzoate as the reactive group was used. In particular, free sulfhydryl groups on both the zymogen and activated forms of a subunit enable them to reversibly bind to the reactive group by forming covalent mercaptide bonds, and are eluted by reducing agents. The b subunit does not bind to the affinity gel and is held to it only through interaction with the a subunit (McDonagh et al., 1976).

Immunoaffinity chromatography using immobilized monoclonal antibodies offers potential one-step purification of many proteins to high purity. In a more recent study by Ikura et al. (1987), monoclonal antibody against bovine plasma FXIII was successfully applied to a one-step purification procedure for this enzyme (specifically the catalytic
units). Purification of human plasma FXIII using specific monoclonal antibodies has been
developed and patented (Hock, 1994).

In the present study, the potential of polyclonal antibody raised in chicken's egg
yolk for use in the one-step immunoaffinity chromatographic purification of bovine
plasma FXIII was investigated. As mentioned above, bovine plasma FXIII was not
commercially available. It was first, therefore, necessary to purify this enzyme in order
that specific antibodies may be produced and collected. Purification of bovine plasma
FXIII using polyclonal antibodies from egg yolk may present an inexpensive and
relatively simple alternative to other methods currently available.
1.2 Objectives

The primary objective of the present study was to develop an immunoaffinity chromatographic method for the purification of bovine plasma FXIII from a source of bovine plasma proteins (Fibrimex) and to assess the potential of using polyclonal antibodies raised in chicken’s egg yolk for this purpose. To do this, a method was developed for the initial purification of the enzyme for the purpose of producing specific antibodies against it. Then, as a secondary objective, this method was compared to the immunoaffinity chromatographic procedures developed. Results were also compared to what has been reported in the literature. Criteria for comparison include purity, yield and activity of enzyme, as well as ease of use.
CHAPTER II
LITERATURE REVIEW

2.1 Enzymatic Modifications of Food Proteins

Food science is the science of food production, processing and utilization. Food technologists invent and reinvent techniques to better achieve the goals of producers, processors, consumers, and regulatory bodies. Well-controlled physical, chemical, or enzymatic modification of proteins in food, and indeed other food constituents can produce or improve desirable functional and/or nutritional properties. Chemical methods for protein modification have been studied extensively and have proven to be effective in many cases (Feeney, 1977, pp. 3-4). However, barriers remain, regarding the use of these methods for food proteins destined for human consumption. This may be due to secondary reaction products, some of which are toxic (Alexandre et al., 1993). In addition, the reagents themselves may be toxic or unacceptable (Colas et al., 1993).

Enzymes are naturally occurring proteins that catalyze specific chemical reactions important to life. The use of enzymes in food processing has been known to man for centuries, and it may overcome the difficulties associated with chemical modifications (Colas et al., 1993).

The use of proteolytic enzymes for protein modification has been widely explored and applied. Renneting of milk casein micelles into curd during the early stages of traditional cheese-making is probably the most familiar case of such use. However, as Dickinson (1997) noted, the action of most proteolytic enzymes typically results in the breakdown of macromolecular structure. For instance, the cleavage of peptide bonds by
pepsin and trypsin leads to lower viscosity of the protein solution, and hence prevents the proper formation of gels (Dickinson, 1997). Nevertheless, these degradative reactions are useful in certain food processing applications such as: body, flavour, and nutrient development in brewing, meat and fish tenderization, soybean milk production, wine clarification, and preparation of protein hydrolysates (Whitaker, 1994, p. 17).

Protein crosslinking enzymes, in contrast, are much less well known and applied. They also provide a natural means for the modification of texture and other attributes in foods. Among them, transglutaminase has in recent years come into the spotlight of manufacturers, consumers and scientists alike.
2.2 Transglutaminase

2.2.1 Catalytic Reactions

Transglutaminase (protein-glutamine : amine γ-glutamyltransferase EC 2.3.2.13) is a family of enzymes which catalyze the acyl transfer reaction between the γ-carboxyamide group of a peptide-bound glutaminyl residue and a primary amine (see Figure 2.1) (Dickinson, 1997). When the ε-amino group of a peptide bound lysine residue acts as substrate, the two peptide chains are covalently linked through an ε-(γ-glutaminyl)lysine bond (or isopeptide bond) (Dickinson, 1997); this is accompanied by the reticulation of the proteins (Chobert et al., 1996). In the absence of amines, transglutaminase catalyzes the hydrolysis of the γ-carboxyamide group of the glutaminyl residue; this results in deamidation (Dickinson, 1997).

According to Dickinson (1997), the rate of crosslinking is dependent on the macromolecular structure of each protein substrate. For instance, reactive glutamine residues generally reside in flexible regions of the polypeptide chain or in regions with reverse turns. Therefore, caseins, for example, would be and are in fact good substrates owing to their flexible structures. On the other hand, as Dickinson (1997) explained, it has been widely reported that globular food proteins such as ovalbumin and β-lactoglobulin are not attacked in their native states. The susceptibility of globular proteins to transglutaminase-induced crosslinking may be increased in several ways: by chemical modification, by disruption of intermolecular disulfide bonds, by conversion into the molten globular state or by adsorption at the oil-water interface (Dickinson, 1997). Other factors affecting the reaction rate are temperature, pH and calcium content (Dickinson, 1997).
According to Seguro et al. (1996), \(\varepsilon-(\gamma\text{-glutaminyl})\)lysine bonds have been found in such raw foods as meats, fish, shellfish, and fish eggs. Isopeptide bonds are also known to occur in many processed foods, such as cooked meats and poultry, kamaboko, and liver, sometimes in greater amounts than in their raw materials. Interestingly, milk and other dairy products contain little or no such bonds (Seguro et al., 1996).
Figure 2.1: Schematic of the Protein Crosslinking Reaction Catalyzed by Transglutaminase

\[
\text{Transglutaminase} \quad \xrightleftharpoons{\text{NH}_3} \quad \text{Polypeptide chain}
\]
2.2.2 Source

In vivo, transglutaminase apparently is quite ubiquitous and plays a variety of vital roles in the life of an organism. Aberrations from the normal functioning of these enzymes may pose many health hazards, many of which have only begun to be understood.

Several types of calcium-dependent transglutaminases, performing various biological functions occur in mammalian tissues and organs, such as plasma, platelet, placenta liver, red blood cells, hair follicles, prostate gland, and skin (De Backer-Royer et al., 1992a; Signorini et al., 1988; Ando et al., 1987; Folk 1980; Folk and Finlayson 1977; Folk and Chung, 1973). They have been demonstrated or suspected to be involved in many important processes including: blood clotting, normal wound healing, growth regulation, maintenance of erythrocyte rigidity, formation of skin and hair, fertilization events, several disease states, retention of placenta, receptor-mediated endocytosis, hormone secretion, and cell differentiation (Ando et al., 1987; De Backer-Royer et al., 1992a).

In particular, human plasma FXIII is an extracellular transglutaminase that catalyzes formation of insoluble fibrin clots during the final step of the blood coagulation cascade. This crosslinking serves to impart mechanical strength to blood clots, anchoring them to the site where the injury has occurred, and rendering them less susceptible to proteolysis (Dickinson, 1997). Also, according to Murthy et al. (1998), highly insoluble neurofibrillary tangles found in brains of humans with Alzheimer’s disease were shown to be degraded by bacterial isopeptidase (Bacillus cereus). Therefore, it was further hypothesized that Alzheimer’s disease may be linked, at least in part, to the up-regulation
of transglutaminase and down-regulation of isopeptidase in brain tissue (Murthy et al., 1998). In addition, prostate transglutaminase produces the vaginal plug by post-ejaculatory clotting of rodent seminal plasma (Ando et al., 1987).

Transglutaminase is also known to exist in fish, fish eggs and shellfish. It has, for instance, been purified from liver tissue of the red sea bream (Pagrus major) (Yasueda et al., 1994) as well as walleye Pollock (Theragra chalcogramma) liver (Kumazawa et al., 1996). Two types of transglutaminase have been isolated from the gills and mantles of Japanese oysters (Crassostrea gigas) (Kumazawa et al., 1997). Two kinds of transglutaminase isoforms were shown to localize in the chorion fraction of the eggs of rainbow trout (Oncorhynchus mykiss) (Fukuda et al., 1998). With respect to processed fish, endogenous transglutaminase is believed to be involved in the setting of fish meat sol to gel at room temperature, imparting the unique texture of fish meat gel (Tsukamasa et al., 1993).

In addition, transglutaminase activity has been reported in invertebrates, and in particular, in the hemolymphs of limulus, lobster, sand crab, and sponge (Tokunaga et al., 1993).

Furthermore, transglutaminase is found in the buds and sprouts of some plants, such as alfalfa, sunflower and pea (Takagi et al., 1986). A calcium-independent transglutaminase, secreted extracellularly by a variant of Streptoverticillum mobaraense has been discovered (Seguro et al., 1996). Lastly, recombinant FXIII has been produced by fermentation of Saccharomyces cerevisiae; this enzyme is identical with platelet FXIII and placental FXIII and has the form α2 (Nielsen et al., 1995).
2.2.3 Characteristics

The characteristics of transglutaminase vary according to its source. Human plasma FXIII, guinea pig liver transglutaminase, and microbial transglutaminase (Streptoverticillum mobaraense var.) have noticeably been the subjects of most studies. Other transglutaminases which have been isolated and characterized to different degrees include: human placental FXIII, human platelet FXIII, human epidermal transglutaminase, human erythrocyte transglutaminase, bovine plasma FXIII, rainbow trout fish egg transglutaminase, and limulus hemocyte transglutaminase. A thorough comparison of the different transglutaminases occurring in nature is beyond the scope of the present discussion. Four enzymes having transglutaminase activity and with distinct molecular structures and other characteristics are presented below to illustrate the variability that exists among these enzymes.

**Human plasma and placental FXIII.** According to McDonagh (1994), human FXIII exist as inactive zymogens and are further divided into extracellular (or plasma) and intracellular transglutaminase. Placental FXIII is an example of intracellular transglutaminase. The building blocks of these enzymes are either one polypeptide, or two nonidentical polypeptides, commonly referred to as the \( a \) and \( b \) subunits. The \( a \) subunit is where the catalytic function resides. It also contains the activation peptide important for the conversion of zymogen to active transglutaminase. One of the nine free sulfydryl groups in \( a \) subunit has been identified as the active center. The \( b \) subunit, in contrast, contains no free sulfhydryl groups but is glycosylated. The subunits are held together by non-covalent forces (McDonagh, 1994).
As McDonagh (1994) described, plasma FXIII (denoted by $a_2b_2$) contains both kinds of subunits, which form a tetrameric molecular complex consisting of two $a$ chains and two $b$ chains held together by strong, non-covalent interactions. This complex, which is found only in plasma, has a molecular weight of 320,000, with the $a$ chain being 82,000 and the $b$ chain 76,500. The isoelectric point of the tetramer is 5.2, and its extinction coefficient is 13.8 cm$^{-1}$ %$^{-1}$. On the other hand, placental FXIII (denoted by $a_2$), contains $a$ subunits only (McDonagh, 1994).

McDonagh (1994) stated that both plasma and placental FXIII, in order to exhibit full enzymatic activity, requires both a proteolytic modification and a conformational alteration (McDonagh, 1994). Figure 2.2 is a schematic depicting the general reactions proposed to be required for enzyme activation of plasma FXIII. Activation of placental FXIII also requires both thrombin and calcium ion but obviously involves no release of $b$ subunits (McDonagh, 1994).

The activation process itself is, nonetheless, still not fully understood. According to McDonagh (1994), although it is presumed that thrombin is the physiologically important proteolytic activator, there are several other enzymes that can also activate FXIII in vitro, including trypsin and papain. High non-physiological concentrations of metal ions (Ca$^{2+}$, sodium chloride, and potassium chloride) will activate the zymogen directly (McDonagh, 1994). Here, the active enzyme is distinguishable from the usual $a_2^*$ form in that it has the activation peptide remained attached to the protein (Ikura et al., 1987).

In addition, in earlier literature, it was often stated, without hints of uncertainties or needs for qualification, that calcium ions, besides inducing the dissociation of the $a$
subunits from the $b$ subunits, are responsible for the conformational changes on the $a$ subunits necessary for expression of activity. Yet, recently it has been suggested that calcium ions, and even other more 'potent' metallic ions do not induce enough conformational changes to expose the active site(s) of the enzyme (Fox et al., 1999).

As McDonagh (1994) explained, although $a_2^*$ is the actual catalytic unit, the function of the $b$ chain is also of interest. In the activation of plasma FXIII ($a_2b_2$) there is a lag phase that is not present in placental FXIII ($a_2$) activation. The combination of purified $b_2$ with $a_2$ chains (platelet transglutaminase) reproduces the lag phase seen with the native $a_2b_2$. These results suggest that the rate-limiting step in the activation of plasma FXIII is the dissociation of $b_2$ from the thrombin-modified complex. In addition, it has been proposed that the $b$ chain may act as a protective carrier for the $a$ chain in plasma FXIII (McDonagh, 1994).
Figure 2.2: Schematic of the Activation of Plasma FXIII Zymogen *In Vivo*

Activation peptides

Calcium ion dependent dissociation

Inactive plasma FXIII zymogen

$\alpha_2\beta_2$

Active enzyme

$\alpha^*_2$

Thrombin dependent activation

$\beta_2$
Microbial transglutaminase. According to Motoki and Seguro (1998), microbial transglutaminase (*Streptoverticillum mobaraense var.*) is a monomeric protein containing 331 amino acid residues (MW of $3.8 \times 10^4$, isoelectric point of 8.9). In contrast to a typical mammalian transglutaminase, it does not require the presence of calcium ions to exhibit enzymatic activity. Incidentally, this property becomes especially important in terms of its applications in food processing, because many common food proteins (e.g. casein) have the tendency to precipitate at relatively low calcium ion concentrations. Also, no proteolytic activation, such as the action of thrombin on FXIII, of the macromolecule is necessary. In addition, the fact the enzyme is secreted into the cultural broth and therefore may be readily purified from the cell materials makes its production for food use much more feasible on a larger commercial scale. Microbial transglutaminase products are now available on the market for those seeking an alternative protein modification agent (Motoki and Seguro, 1998).

Fish Egg Transglutaminase. Polyclonal antibodies against guinea pig liver transglutaminase (subunit MW ~80 kDa) have been found to react with a protein (SDS-PAGE bands transferred onto nylon membranes) in the chorion fraction from the eggs of rainbow trout (*Oncorhynchus mykiss*) (Fukuda et al., 1998). The apparent molecular mass of this protein was estimated to be about 70 kDa, which is similar to that of rat liver transglutaminase. It was not immediately clear if this protein was responsible for the transglutaminase activity observed (in the chorion fraction) in this study.
2.2.4 Applications in Food Technology

The strong covalent bonds resulting from the reaction catalyzed by transglutaminase are responsible for the changes of the molecules involved and ultimately the product being treated with transglutaminase. Transglutaminase is the only crosslinking enzyme that is currently available for catalyzing covalent bond formation between protein molecules on a commercial scale (Dickinson, 1997).

"Using this enzyme, food technologists have the opportunity to generate novel gel-like network structures from low-viscosity protein solutions and dispersions, as well as from various colloidal systems, such as those containing protein-coated emulsion droplets or protein-coated gas bubbles" (Dickinson, 1997).

Many common food proteins have been identified as substrates for the transglutaminase-catalyzed crosslinking reaction. Each of pea legumin (Larré et al., 1993b), gliadin (Larré et al., 1993a), beef actomyosin (Kim et al., 1993), and fish actomyosin (Joseph et al., 1994) has been successfully polymerized using guinea pig liver transglutaminase. In particular, low temperature crosslinking and gelation of beef actomyosin has been demonstrated and may prove to be useful for meat restructuring or forming without the requirement for freezing or cooking to maintain product integrity (Nielsen et al., 1995). Transglutaminase (guinea pig liver) modified gliadin formed an emulsion with better resistance to coalescence than chemically (acylation with citraconic anhydride) treated gliadin. This may be related to the presence of high MW crosslinked
materials which stabilize the emulsion interface, in this case, hexadecane/water (Larré et al., 1993a). Motoki et al. (1987) produced heterologous polymers containing milk casein and soybean globulin have been produced in the laboratory using, again, guinea pig liver transglutaminase. The heteropolymers thus formed had significantly higher solubility than the nonpolymerized mixture at certain pHs and produced slightly more stable emulsions as well as higher emulsion activity (indicated as emulsion turbidity) (Motoki et al., 1987).

Human placental FXIII has been shown to polymerize a range of soybean proteins, including the 7S and 11S (soybean) globulins and their subunits (Siepaio and Meunier, 1995). The same enzyme has also been shown to crosslink myosin, actin, (De Backer-Royer et al., 1992b) and spinach rubilose bisphosphate carboxylase (RuBisCO) (and the subunits thereof) (Siepaio and Meunier, 1995). In particular, De Backer-Royer et al. (1992b) showed that myosin and soybean 7S globulin gels were formed by species of high MW and nonpolymerized proteins for globulins and only by high MW species for myosin. In both cases, isopeptide bonds (whose formation was catalyzed by the added human placental FXIII) were believed to be involved (De Backer-Royer et al., 1992b).

Studies using bovine plasma FXIII have demonstrated that this enzyme is capable of crosslinking myosin to soy protein, casein or gluten (Kurth and Rogers, 1984). Bovine plasma protein (containing bovine plasma FXIII) treatment of Pacific whiting surimi has resulted in the formation of protein crosslinks, which contributes to gel strength enhancement (Seymour et al., 1997).

The crosslinking of a variety of protein substrates catalyzed by the microbial transglutaminase derived from Streptoverticillum mobaraense var. has been well
documented. These substrates include soy protein, casein, whey protein, egg yolk and egg white proteins (Dickinson, 1997). In particular, microbial transglutaminase may be useful in meat binding and restructuring (Ohr, 1999).

"In contrast to traditional meat binding agents, microbial transglutaminase creates cross-linked proteins, forming 'a bond similar to that created by nature', therefore offering an alternative to binding agents that may adulterate meat products with a standard of identity" (Ohr, 1999).

Ohr (1999) also stated that nutritional benefits might be indirectly derived from the use of this enzyme to enhance the texture and moisture retention of reduced-fat and reduced-salt meat products. Indeed, the enzyme is now approved for use in foods in Japan and some European countries. In Japan, microbial transglutaminase is currently used in surimi, meat, soy protein and noodle products. Microbial transglutaminase has been found to be 'generally recognized as safe' in the U.S. and is awaiting USDA approval (Ohr, 1999). Motoki and Seguro (1998) reviewed the use of transglutaminase for food processing, with emphasis on the current and potential applications of microbial transglutaminase in meat, fish, dairy, soybean, wheat and other products.

Colas et al. (1993) used guinea pig liver transglutaminase to covalently attach glycosyl units to glutamine residues of legumin and β-gliadins. The solubility of the neoglycoproteins thus produced was markedly increased over that of native proteins in the range of their isoelectric points. However, this effect was much less pronounced for
pHs far from the pl. For pH values below 5.0, the solubility of glycosylated β-gliadins was even slightly lower than that of native β-gliadins (Colas et al., 1993).

Using guinea pig liver transglutaminase, Ikura et al. (1981) incorporated L-methionine ethyl ester into α_{s1}- and β-caseins as well as soybean 7S and 11S proteins. The methionine content of these proteins was increased by as much as 350%. With wheat gluten, incorporation of L-lysine was tested and a 5.1-fold increase in lysine content was observed. These authors suggested that transglutaminase could be a useful tool for improving the amino acid composition and therefore the nutritive value of food proteins by the covalent attachment of limiting essential amino acids (Ikura et al., 1981).

Besides crosslinking reactions, transglutaminase catalyzes the hydrolysis of the γ-carboxyamide group (deamidation) of the glutaminyl residue. According to Alexandre et al. (1993), chemical deamidation in mild conditions (acid concentration, temperature) has long been recognized as a powerful tool to solubilize gluten proteins in water. This is explained by the negative charge increase resulting from carboxylic group (COO\(^{-}\)) formation. However, at higher rate of deamidation (>20%), acidic treatment generally induced a partial hydrolysis of the proteins but it was difficult to control the level of hydrolysis. From a general point of view, the use of enzymes for protein modification provides advantages including mostly the mild reaction conditions and the specificity of the reaction. In the case of food applications, enzymatic reactions may present advantages compared to the use of chemical reagents, which can lead to toxic contaminants through secondary reactions. Wheat proteins are good substrates for transglutaminase-catalyzed deamidation because they are rich in glutaminyl residues (30% of the total residues) and poor in lysyl residues (1%). Also, both chemical and enzymatic deamidation considerably
improves the solubility of gliadins in the pH range of 5-9 (Alexandre et al., 1993). Alexandre et al. (1993) optimized the use of bovine plasma FXIII for modifying gluten proteins.
2.3 Bovine Plasma FXIII

The blood of slaughtered livestock is severely underutilized as a source of protein for humans, despite its high protein quality and high dietary iron content (Guzman et al., 1995). The potential for the utilization of blood plasma proteins in meat, bakery products and other food products to effect changes in functional properties has been recognized for some time (Howell and Lawrie, 1984). One vehicle through which blood plasma proteins contribute to these changes is its role as a meat binder.

In the present study, Fibrimex (FNA Foods, Inc., Calgary, AB, Canada) was used as a source of bovine plasma FXIII. Fibrimex may be used to facilitate gel formation in the manufacture of restructured meat products without the application of heat (Boles and Shand, 1998). It is marketed as a meat-binding process during which its active ingredients (fibrinogen, thrombin, and bovine plasma FXIII) react and act, in a manner analogous to blood clotting, to induce desirable binding effects (Wijngaards and Paardekooper, 1997). The resulting products, in contrast to those prepared using thermal gelation, may not only be sold either precooked or frozen but also in the chilled, raw state (Boles and Shand, 1998). Also, less discoloration and oxidative rancidity are associated with them (Boles and Shand, 1998).

Due to the crucial role that human plasma FXIII plays in human health, studies concerning this enzyme are widely found in the literature. In contrast, only very few scientific articles pertaining specifically to bovine plasma FXIII are available. Bovine plasma FXIII is a calcium-dependent extracellular transglutaminase, which exists, in bovine plasma as the inactive zymogen \(a_2b_2\) (Ikura et al., 1987). Activation by calcium and thrombin are required for the expression of catalytic activity. Ca-independent
activation can also occur with bovine plasma FXIII (Ikura et al., 1987). Generally, when bovine plasma FXIII is discussed, it is usually taken to be analogous to its human counterpart (as in Alexandre et al., 1993). As noted earlier, a number of food proteins (myosin, soy protein, casein, and gluten) are substrates of bovine plasma FXIII.
2.4 Enzyme Purification

2.4.1 Strategies

The subject of the present study is enzyme purification. In general, difficulties remain in finding optimum conditions for protein extraction and sample pretreatment as well as in choosing suitable methods for monitoring protein and biological activity. In terms of the choice of material, one must consider the concentration of target protein, the presence of interfering compounds, and cost.

The objectives of a scheme for the purification of an enzyme or other protein may be any or all of the following, namely, high degree of purity and recovery of activity of the end product, reproducibility within and between laboratories, potential for scaling up or down, ease of use, and cost (Scopes, 1994, p. 310).

According to Ersson et al. (1989), after the initial fractionation, the logical sequence of chromatographic steps that follow, would be, to start with more ‘robust’ techniques (e.g., ion-exchange or hydrophobic interaction chromatography), which combine high capacity, simplicity and low cost of raw material. Protocols are often finished with a gel filtration step. In addition, it is advisable to design the sequence in such a way that buffer changes and concentration steps are avoided as much as possible (Ersson et al., 1989).

2.4.2 Ion-exchange Chromatography

According to Pharmacia Biotech Inc. (1991), the technique of ion-exchange chromatography is based on the reversible adsorption of charged solute molecules (e.g., proteins) to an immobilized ion exchanger of opposite charge. It is essential for the ion-
exchanger to be brought to equilibrium, in terms of pH and ionic strength to enable the binding of the desired solute molecules. After sample has been applied and unbound substances washed off using the starting buffer, substances still bound can be removed from the column by introducing the eluting buffer that weakens ionic bonding of the solute molecules. The desired elution conditions may be achieved by raising the pH or the ionic strength of the eluting buffer as step-wise or continuous gradient. When all substances have been desorbed, the ion-exchanger is once again brought to the starting conditions for the next purification. (Pharmacia Biotech Inc., 1991, pp. 6-7). For a review of the principles of the elution of bound proteins during ion-exchange chromatography, readers are directed to Scopes (1994, pp. 146-171).

As Pharmacia Biotech Inc. (1991) explained, in principle, one can choose whether to bind the substances of interest and allow the contaminants to pass through the column, or to bind the contaminants and allow the substance of interest to pass through. Generally, the first method is more useful since it allows a greater degree of fractionation and concentrates the substance of interest (Pharmacia Biotech Inc., 1991, p. 7).

Automated systems such as Fast Protein Liquid Chromatography (FPLC) from Pharmacia Biotech Inc. combine state-of-the-art separation media, instrumentation, hardware and software making them the choice for protein separation.

2.4.3 Protein G Affinity Chromatography

Protein G is a bacterial cell wall protein isolated from group G streptococci (Akerstrom et al., 1995). Protein G binds to most mammalian immunoglobulins through their Fc regions. Since Protein G also contains albumin and cell surface binding sites,
these sites are eliminated (e.g., genetically deleted during cloning) in commercial preparation to reduce non-specific binding when Protein G is used to purify immunoglobulins (Pierce Chemical Co., 1988). Protein G has strong binding affinity for polyclonal immunoglobulins from a variety of sources, including human, pig, and cow (Pharmacia Biotech Inc, 1993). This property was utilized in this study to remove bovine immunoglobulins during the purification of bovine plasma FXIII.

2.4.4 Immunoaffinity Chromatography Using Yolk Immunoglobulins

Affinity chromatography refers to the use of a ligand, which specifically interacts with the desired protein for the purpose of its purification (Scopes, 1994, p. 187). The ligand used in immunoaffinity chromatography is an immunoglobulin to the protein being purified or vice versa.

According to Godfrey (1997), immunoglobulins (or antibodies) are molecules found in the serum and secretions of animals; they are usually produced in response to, and are specific for, foreign substances or antigens. Production of immunoglobulins may be stimulated by immunization with purified antigen preparations (immunogens). The resulting antibodies are referred to as polyclonal antibodies (pAbs) since they consist of a heterogenous population of immunoglobulins, of which a few clones will be directed towards the antigenic sites (epitopes) on the immunogen (Godfrey, 1997, p. 141). Monoclonal antibodies (mAbs) are a homogenous immunoglobulin population and are secreted by clones of a cell constructed by the fusion of an immortal myeloma cell with an antibody secreting cell (splenocyte or lymphocyte), from an immunized animal, to produce a hybridoma cell (Godfrey, 1997, p. 141-142).
Each type of antibodies has its advantages and disadvantages. For example, monoclonal antibodies may be produced in large quantities at much higher purity than polyclonal antibodies and with permanency of supply (Scopes, 1994, p. 209). On the other hand, equipment and expertise required for their production are much more demanding than polyclonal antibody production (Scopes, 1994, p. 209).

Immunoaffinity chromatography enables the isolation of high purity products from complex fluids using a one-step separation (Kim and Li-Chan, 1998). Adsorption, washing, and elution steps must be optimized carefully because of the extreme lability and sensitivity of many enzymes. In most applications, according to Kim and Li-Chan (1998), polyclonal antibodies from mammalian antisera or monoclonal antibodies have been used to construct the immunosorbent. However, compared to these sources of antibodies, yolk immunoglobulins have several advantages: high yields of antibody, ease of collecting eggs, elimination of need for bleeding, and lower cost compared with monoclonal antibodies (Kim and Li-Chan, 1998). In addition, the possibility of cross-reactivity problems (between species) is minimized and, in some cases, the purification of antibodies is considerably simplified (Li-Chan et al., 1998).

Still, as Ehle and Horn (1990) explained, three main problems are associated with the application of immunoaffinity chromatography for enzyme purification: denaturation of enzyme activity during adsorption and/or elution, antigenicity of target protein, and cross-reactivity (between protein) of the antibodies used. For example, due to the high specificity and high affinity of the antibodies to the enzyme antigen, harsh conditions, such as the use of pH extremes, chaotropic ions, high concentrations of urea and guanidine hydrochloride are often required to elute the bound antigen. This is
exacerbated by the presence of proteinases, especially when crude extracts are used as starting material. Proteinases may degrade the immunosorbent as well as reduce enzyme activity (Ehle and Horn, 1990).

Moreover, according to Scopes (1994), proteins differ in their ability to elicit antigen production in the host being immunized. For instance, antibodies against a highly antigenic impurity in a protein preparation used for immunization may be produced in much greater abundance compared to antibodies against the purified protein. This effect may be worsened if the purified protein is a poor antigen itself (Scopes, 1994, p. 206).

To improve the specificity of polyclonal antibody against a target protein, further purification of the antibody preparation may be desirable. This may be achieved through the adsorption of specific antibody using its antigen, or alternatively, through the removal of unwanted antibodies using proteins with which the antibody preparation is suspected to cross-react with (referred to as subtractive immunoaffinity chromatography; see Lee et al. (1995) for an example of its use). Both of these techniques were employed in the present study.

Finally, other strategies are available for the recovery of an active undamaged enzyme and these include: selection of antibody affinity, careful selection of desorption conditions and optimization of elution conditions, use of proenzyme purification as well as use of immunoligates against enzyme complexes (Ehle and Horn, 1990).
CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Fibrimex was obtained as a gift from FNA Foods, Calgary, AB, Canada. DEAE Sepharose CL-6B, pre-packed RESOURCE Q columns, HiTrap affinity columns (1 ml), pre-packed PD-10 Sephadex G-25 columns (for buffer exchange), 10-15% PhastGel gradient gels, SDS buffer strips and Coomassie Blue dye were purchased from Pharmacia Biotech Inc., Baie d'Urfé, PQ. Glass columns and Econo-Column Flow Adaptor for low-pressure anion-exchange chromatography were purchased from Bio-Rad Laboratories Ltd., Mississauga, ON. ImmunoPure (G) Immobilized Protein G was purchased from Pierce Chemical Co., Rockford, IL. Actigel ALD-Superflow resins, ALD Coupling Solution, and ActiSep were purchased from Sterogene Bioseparations, Arcadia, CA. Dithiothreitol, Tris (Ultrapure), and thrombin were purchased from ICN Biomedicals Canada Ltd., St. Laurent, PQ. Monodansylcadaverine, acetylated casein and High-range and Wide-range SigmaMarkers (molecular weight markers) were purchased from Sigma Chemical Corporation, St. Louis, MO. Alkaline Phosphatase-conjugated AffiniPure Rabbit Anti-Chicken IgY (IgG) (H+L) and alkaline phosphate substrate tablets were purchased from Sigma Chemical Corporation, St. Louis, MO. All other chemicals were of reagent grade, except for glycine, which was of tissue culture grade.
3.2 Overview of Methods

Figure 3.1 is a flow-chart showing the major elements of the methods used in the present study. The following is a brief summary of these elements. Individual procedures are presented in Sections 3.3-3.7. Refer to legends of tables and figures in Results and Discussion for additional details.

**Immunization and extraction of crude (polyclonal) IgY.** Laying hens were immunized with partially purified bovine plasma FXIII obtained using ion-exchange chromatography. In due course, eggs were collected, from which crude IgY were extracted.

**Preparation of immobilized bovine plasma FXIII as an antigen for the immunoaffinity isolation of its specific IgY.** Bovine plasma FXIII was partially purified from Fibrimex using anion-exchange chromatography followed by Protein G affinity chromatography. Heat-induced precipitation of fibrinogen and subsequent buffer-exchange (via dialysis) into the ion-exchange chromatography starting buffer were the only sample preparation steps employed for the chromatographic preparation. Bovine plasma FXIII-rich material thus obtained was immobilized onto Actigel ALD-Superflow resins which were packed into a column for the immunoaffinity isolation of bovine plasma FXIII-specific IgY.

**Isolation of bovine plasma FXIII-specific IgY.** Crude IgY preparations were loaded onto the column containing immobilized bovine plasma FXIII. Alternatively, they were loaded onto a column containing immobilized BSA (subtractive immunoaffinity chromatography). The unbound fractions collected were then loaded onto the column containing immobilized bovine plasma FXIII. In each case, specific IgY were eluted and
then immobilized onto Actigel ALD-Superflow resins which were packed into a column for the one-step immunoaffinity purification of bovine plasma FXIII from Fibrimex.

Imunoaffinity purification of bovine plasma FXIII. The two immunoaffinity columns thus produced were used for the purification of bovine plasma FXIII, results from which were evaluated.

Analyses. Protein concentrations were measured spectrophotometrically and/or by means of commercially available protein assay kits. Factor XIII enzymatic activity was determined using the method of Takagi et al. (1986) with slight modifications. Enzyme-linked immunosorbent assays (ELISAs) were performed to qualitatively evaluate the activity of the crude IgY concentrates against the antigens produced. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and mercaptoethanol (reducing SDS-PAGE) was performed to reveal the protein composition, the purity of a given protein solution, and thus the progress of purification.
Figure 3.1: Flow-Chart of Methods

- Immunization & Crude IgY Preparation
- Purification of Specific IgY
- Immunoaffinity Chromatographic Purification of Bovine Plasma FXIII

Ion-exchange-purified Bovine plasma FXIII

...
3.3 Immunization of Hens and Extraction of Crude (Polyclonal) IgY

3.3.1 Partial Purification of Bovine Plasma FXIII

Bovine plasma FXIII was partially purified by Karoline Lee (Department of Food Science, UBC) using ion-exchange chromatography; this was the source of antigens for the immunization of laying hens. Enzyme activity rich fractions from three ion-exchange chromatography experiments (Table 3.1) were pooled to give the final extract used for immunization. This extract is denoted hereinafter by LEE ("Lee’s Enzyme Extract").

Fibrimex was fractionated on DEAE-Sepharose CL-6B resins using three different elution protocols in the three experiments. However, in all cases, the running buffer was 0.05M PB (K\(^+\)) + 1mM EDTA at pH 7.5. It was found that eluted activity peaked at about 0.11M NaCl in the running buffer when continuous gradient elution was used. Upon reducing SDS-PAGE, the fractions bearing the highest bovine plasma FXIII activity showed one (~ 63-68 kDa) or two major bands (~ 63-68 and ~100 kDa). It may be speculated that the protein band corresponding to a molecular weight of ~63-68 kDa represents bovine plasma FXIII (co-migrating \(a\) and \(b\) subunits). However, due to the proximity of this band to the theoretical location of BSA (66 kDa) and the low specific enzymatic activities associated with these ‘active’ fractions, it remains possible that the band at ~63-68 kDa represents a mixture of BSA and bovine plasma FXIII.
Table 3.1: Summary of Anion-exchange Chromatography

Experimental Conditions, for the Partial Purification of Bovine Plasma FXIII Used for Immunization

<table>
<thead>
<tr>
<th>Running Buffer</th>
<th>Starting Material</th>
<th>Mode of Gradient Elution</th>
<th>Eluent(s) ([NaCl] in Running Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05M PB + 1mM EDTA at pH 7.5</td>
<td>HFSN(^a)</td>
<td>Stepwise</td>
<td>0.12M</td>
</tr>
<tr>
<td>0.05M PB + 1mM EDTA at pH 7.5</td>
<td>HFSN(^a)</td>
<td>Continuous</td>
<td>0 – 100% 0.5M</td>
</tr>
<tr>
<td>0.05M PB + 1mM EDTA at pH 7.5</td>
<td>HFSN(^a)</td>
<td>Stepwise</td>
<td>0.10M</td>
</tr>
</tbody>
</table>

\(^a\)HFSN = “Heated Fibrinex Supernatant” (see Section 3.4.1)
3.3.2 Immunization of Hens and Preparation of Crude IgY Extract

Immunization procedures were performed as described by Li-Chan et al. (1998) by staff at the UBC Animal Care Centre, with the assistance of Karoline Lee and Angela Kummer from the Department of Food Science. Laying hens were first (Day 1) injected with LEE (at 1 mg/ml) suspended in Freund's complete adjuvant. On Day 14 and again on Day 35, LEE (at 1 mg/ml) suspended in Freund's incomplete adjuvant was administered as boosters. Injections were done with a 3 cc Luerlock syringe (with a 20 G 1 ½ needle). The eggs, after they were collected, were allowed to sit for at least 1 month before they were processed (Kwan et al., 1991). The yolk was diluted 1:9 with distilled water, that is 1 volume in a total of 10 volumes. The yolk mixture was acidified to pH 5.2 with 0.1N HCl, left overnight at 4°C, and the next day centrifuged at 16,000 x g for 25 min at 4°C. The supernatant was collected, filtered through Whatman #4 filter paper and glass wool, measured and 19% solid ammonium sulphate was added, stirred until dissolved, and the mixture was placed at 4°C overnight. Everything was centrifuged again as above, the supernatant discarded, and the pellet was taken up in 0.1M NaCl in 0.05M PB + 1mM EDTA at pH 7.5. This crude IgY concentrate was frozen at –28°C until use.
3.4 Bovine Plasma FXIII Purification for the Isolation of Its Specific IgY

3.4.1 Sample Preparation for Ion-exchange Chromatography

One 1-kg bag of Fibrimex was removed from frozen storage (-25°C) and was left to thaw at 4°C overnight. The liquid was decanted; the remaining solid material was centrifuged at 4°C at 10,400 × g for 20 min. The supernatant was combined with the decanted liquid, which was then heated in a 60°C water-bath with constant stirring with a glass rod until the liquid temperature reached 56°C, then held there for 4 min, according to Loewy et al. (1957). The coagulated material was immediately cooled in an iced water-bath for 30 min, followed by centrifugation at 4°C at 10,400 × g for 20 min. The pellet was discarded; the supernatant was frozen at −18°C as ca. 50-ml aliquots. Sodium azide was added at 0.02% as a preservative. This material is denoted hereinafter by HFSN ("Heated Fibrimex Supernatant").

In one experiment, Fibrimex was precipitated by 20% saturated ammonium sulphate overnight at 4°C (20% saturated ammonium sulphate precipitation constituted the first step in a series of such precipitations used by Lorand and Gotoh (1970) in their purification procedures). Afterwards, the precipitated mass was centrifuged at 4°C at 10,400 × g for 20 min; the supernatant was discarded and the pellet was heated at 56°C for 4 min, cooled and centrifuged to obtain the supernatant. This supernatant was refrigerated at 4°C until use.

3.4.2 Anion-exchange Chromatography

Thawed HFSN aliquot was dialyzed (at 4°C) against the starting buffer for 36-48 hour with 4 changes of buffer. Its protein concentration was then determined using Biuret
protein assay. The starting material was subsequently diluted to the loading concentration with the starting buffer. Where FPLC was used, all buffers as well as the starting material were filtered (0.45µ or finer) to remove any particulates present. The starting material was fractionated on pre-packed RESOURCE Q resin in continuous mode using stepwise and/or continuous gradient elution method on the FPLC system (Pharmacia Biotech Inc., Baie d’Urfé, PQ). Alternatively, DEAE-Sepharose CL-6B resins packed in to a glass column was used with a pump. Enzyme activity assay and reducing SDS-PAGE were performed on selected fractions and/or the starting material. The conditions of the individual chromatography runs are found in Table 3.2 and Table 3.3.
Table 3.2: Summary of Anion-exchange Chromatography

Experimental Conditions on RESOURCE Q Resin

<table>
<thead>
<tr>
<th>Running Buffer</th>
<th>Starting Material</th>
<th>Mode of Gradient Elution</th>
<th>Eluent(s) ([NaCl] in Running Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025M Tris-HCl + 1mM EDTA at pH 8</td>
<td>HFSN(^a)</td>
<td>Continuous</td>
<td>0 – 100% 0.5M</td>
</tr>
<tr>
<td>0.025M Tris-HCl + 1mM EDTA at pH 8</td>
<td>HFSN(^a)</td>
<td>Continuous and Step-wise</td>
<td>0 – 50% 0.5M, 0.5M</td>
</tr>
<tr>
<td>0.025M Tris-HCl + 1mM EDTA at pH 8</td>
<td>HFSN(^a)</td>
<td>Stepwise</td>
<td>0.12M, 0.5M</td>
</tr>
</tbody>
</table>

\(^a\)HFSN = “Heated Fibrinex Supernatant” (see Section 3.4.1)
### Table 3.3: Summary of Anion-exchange Chromatography

#### Experimental Conditions on DEAE-Sepharose CL-6B Resin

<table>
<thead>
<tr>
<th>Running Buffer</th>
<th>Starting Material</th>
<th>Mode of Gradient Elution</th>
<th>Eluent(s) ([NaCl] in Running Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025M Tris-HCl + 1mM EDTA at pH 8</td>
<td>HFSN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Stepwise</td>
<td>0.06M, 0.10M, 0.15M, 1M</td>
</tr>
<tr>
<td>0.025M Tris-HCl + 1mM EDTA at pH 8</td>
<td>HFSN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Stepwise</td>
<td>0.04M, 0.1M, 1M</td>
</tr>
<tr>
<td>0.025M Tris-HCl + 1mM EDTA at pH 8</td>
<td>HFSN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Stepwise</td>
<td>0.04M, 0.06M, 0.08M, 0.10M, 1M</td>
</tr>
<tr>
<td>0.025M Tris-HCl + 1mM EDTA at pH 8</td>
<td>HFSN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Stepwise</td>
<td>0.04M, 0.12M, 1M</td>
</tr>
</tbody>
</table>

<sup>a</sup>HFSN = “Heated Fbrimex Supernatant” (see Section 3.4.1)
3.4.3 Protein G Affinity Chromatography

Enzyme-activity rich fractions obtained by anion-exchange chromatography were subjected to Protein G affinity chromatography to remove the bovine immunoglobulins (Ig) present. Only bovine Ig should bind to the chromatography resins.

Separations were performed on either pre-packed HiTrap Protein G column, or ImmunoPure (G) Immobilized Protein G Gel (packed into a column) according to the manufacturer’s recommendations. The resins were first equilibrated with the starting buffer (0.2M acetate buffer at pH 5 for ImmunoPure (G) Immobilized Protein G Gel, and 25mM Tris-HCl + 1mM EDTA at pH 7 in the case of HiTrap Protein G column). Samples were loaded onto the column with a pump, followed by washing with the starting buffer until effluent absorbance at 280 nm was around or below 0.02. The unbound fractions (containing bovine plasma FXIII) were pooled for subsequent experiments. The bound bovine Ig was eluted with 0.1M Glycine-HCl at pH 2.7; eluted fractions were neutralized with 1M Tris-HCl at pH 9. Enzyme activity assay and reducing SDS-PAGE were performed on selected fractions and starting material.

3.4.4 Purification and Immobilization of Bovine Plasma FXIII

Bovine plasma FXIII rich fractions from a single ion-exchange experiment were pooled and subjected to Protein G affinity chromatography using pre-packed HiTrap Protein G column. The unbound fractions thus obtained were pooled, dispensed into dialysis tubing (Fisher Scientific Ltd., Fairlawn, NJ; MWCO: 6-8,000), and sealed. Sucrose was then applied to the surface of the tubing. This served to concentrate the sample by removing some of the water. The concentrated material was then buffer-
exchanged via size-exclusion chromatography (PD-10 Sephadex G-25 column) into 0.1M Phosphate buffer (PB) at pH 7. This process also removed any sucrose and Tris salts present. It was found that little protein binding was achieved in the subsequent immobilization step if dialysis was used instead.

The buffer-exchanged material (obtained by size exclusion chromatography), denoted hereinafter by PGUB ("Protein G Unbound"), was immobilized onto Actigel ALD-Superflow support according to the manufacturer's recommendations. PGUB (7 ml at 1.6 mg protein/ml) was added to 3 ml of Actigel ALD-Superflow previously washed exhaustively with 0.1M PB at pH 7. Manufacturer-supplied coupling buffer (1 ml, corresponding to 10% of the combined volume of PGUB and Actigel ALD-Superflow resins used) was then added to initiate immobilization. The mixture was gently tumbled at room temperature for 1 hour then at 4°C for 16 hr. The mixture was packed by gravity into an empty PD-10 column (d = 1.25 cm, Pharmacia Biotech Inc.). A series of buffers (Table 3.4) was then passed through the column; the effluent at each step was collected and its absorbance at 280 nm was measured. Afterwards, the gel was immediately washed with 25 ml of 0.1M PB at pH 7. The column was stored at 4°C between uses. Endcapping of unreactive aldehyde groups on Actigel ALD-Superflow described in the manufacturer's instructions was omitted.
Table 3.4: Wash Schedule Employed at the End of Bovine Plasma FXIII Immobilization

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Volume</th>
<th>No. of Passes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M PB at pH 7</td>
<td>5 ml</td>
<td>1</td>
</tr>
<tr>
<td>0.5M NaCl in 0.1M PB at pH 7</td>
<td>5 ml</td>
<td>Until absorbance at 280 nm less than 0.02</td>
</tr>
<tr>
<td>0.05M Glycine-HCl at pH 2.8</td>
<td>5 ml</td>
<td>1 (Incubate for 10 min)</td>
</tr>
</tbody>
</table>
3.5 Isolation of Bovine Plasma FXIII-specific IgY

3.5.1 Immunoaffinity Purification of IgY against Bovine Plasma FXIII

One-step method. Thawed crude IgY extract (see Section 3.3.2) was buffer-exchanged (PD-10 Sephadex G-25 column) into 0.14M NaCl in 0.01M PB at pH 7. This was diluted to total protein concentration of about 1.5 mg/ml with 0.14M NaCl in PB at pH 7 before being loaded onto the immunoaffinity column prepared as discussed in Section 3.4.4 (4.9 mg PGUB/ 3 ml resins). The column was then washed with 0.01M PB at pH 7 to remove any unbound or loosely bound IgY. The bound IgY were eluted by 0.05M glycine-HCl at pH 2.8, immediately neutralized using 0.5M PB at pH 8 and stored at 4°C. The eluted fractions from a total of 13 such chromatography runs, performed over a period of seven days, were pooled (total volume about 275 ml), dispensed into dialysis tubing (Fisher Scientific Ltd., Fairlawn, NJ; MWCO: 6-8,000), and sealed. Sucrose was then applied to the surface of the tubing. The concentrated material (~15 ml) was then exchanged via a PD-10 column into 0.1M phosphate buffer (PB) at pH 7. This, denoted hereinafter by SIGY1 (“Specific IgY One-step”), was the source of specific antibodies for the construction of Immunoaffinity Column I (IAC-I) (See Section 3.5.2).

Subsequent immunoaffinity purification of bovine plasma FXIII using IAC-I revealed the possible presence of IgY cross-reacting with BSA in SIGY1. Therefore, the immunoaffinity purification of specific-IgY was repeated but the crude IgY was first put through an immobilized BSA column to remove the cross-reacting IgY (subtractive immunoaffinity chromatography), as described in the following section.

Two-step method. Subtractive immunoaffinity chromatography was first performed. Specifically, crude IgY extract was buffer-exchanged into 0.14M NaCl in
0.01M PB at pH 7 via a PD-10 column. This was loaded onto an immunoaffinity column containing immobilized BSA (15.8 mg BSA/5 ml Actigel ALD-Superflow resins). The column was then washed with 0.14M NaCl in 0.01M PB at pH 7 to obtain the unbound or loosely bound IgY. The bound IgY, containing antibodies against BSA, were eluted by 0.1M glycine-HCl at pH 2.3. Eluted fractions were neutralized with 0.5M Tris at pH 9 and stored at 4°C (typically until the following day when it was used in the second purification step).

As a second step for the isolation of bovine plasma FXIII-specific IgY, the unbound fractions obtained in the first step were pooled and loaded onto the immunoaffinity column prepared as discussed in Section 3.4.4 (4.9 mg PGUB/3 ml resins). The column was then washed with 0.14M NaCl in 0.01M PB at pH 7 to remove any unbound or loosely bound IgY. The bound IgY was eluted by 0.05M glycine-HCl at pH 2.8 followed by 0.1M glycine-HCl at pH 2.3. Eluted fractions were neutralized with 0.5M PB at pH 8 and stored at 4°C. The fractions eluted with 0.05M glycine-HCl at pH 2.8 from a total of 14 such chromatography runs, performed over a period of 10 days, were pooled (volume totaled about 150 ml), dispensed into dialysis tubing (Fisher Scientific Ltd., Fairlawn, NJ; MWCO: 6-8,000), and sealed. Sucrose was then applied to the surface of the tubing. The concentrated material (~10 ml) was then buffer-exchanged via a PD-10 column into 0.1M phosphate buffer (PB) at pH 7. This, denoted hereinafter by SIGY2 ("Specific IgY Two-step"), was the source of specific antibodies for the construction of Immunoaffinity Column II (IAC-II) (See Section 3.5.2).
3.5.2 Immobilization of Specific IgY

Immunoaffinity Column I (IAC-I). SIGY1 (21 ml at 0.26 mg IgY/ml) was added to 3 ml of Actigel ALD-Superflow previously washed exhaustively with 0.1M PB at pH 7. Manufacturer-supplied coupling buffer (2.4 ml, corresponding to 10% of the combined volume of SIGY1 and Actigel ALD-Superflow resins used) was then added to initiate immobilization. The mixture was gently tumbled at room temperature for 1 hour then at 4°C for 16 hr. The mixture was packed by gravity into an empty PD-10 column ($d = 1.25$ cm, Pharmacia Biotech Inc.). A series of buffers (Table 3.5) was then passed through the column; the effluent at each step was collected and its absorbance at 280 nm was measured. Afterwards, the gel was immediately washed with 25 ml of 0.1M PB at pH 7. Endcapping of unreactive aldehyde groups on Actigel ALD-Superflow described in the manufacturer’s instructions was omitted. The column was stored at 4°C between uses.
Table 3.5: Wash Schedule Employed at the End of Antibody Immobilization

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Volume</th>
<th>No. of Times</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M PB at pH 7</td>
<td>5 ml</td>
<td>1</td>
</tr>
<tr>
<td>0.5NaCl in 0.1M PB at pH 7</td>
<td>5 ml</td>
<td>Until absorbance at 280 nm less than 0.02</td>
</tr>
<tr>
<td>0.05M Glycine-HCl at pH 2.8</td>
<td>5 ml</td>
<td>1 (Incubate for 10 min)</td>
</tr>
</tbody>
</table>
**Immunoaffinity Column II (IAC-II).** The method for immobilizing SIGY2 (15 ml at 0.18 mg/ml) was identical to that for SIGY1. However, the volume of Actigel ALD-Superflow resins was reduced to 2 ml. Coupling was therefore initiated by the addition of 1.68 ml of the manufacturer-supplied coupling buffer (corresponding to 10% of the combined volume of SIGY2 and Actigel ALD-Superflow resins used).
3.6 Immunoaffinity Purification of Bovine Plasma FXIII

HFSN was used as the source for the isolation of bovine plasma FXIII using the immunoaffinity columns produced by the above procedures. Frozen HFSN was thawed and buffer-exchanged (PD-10 Sephadex G-25 column) into 0.05M Tris-HCl at pH 7. 0.01M PB at pH 7 and 0.5M PB at pH 8 were initially used as the starting and neutralizing buffer respectively. However, it was found that even after the eluted fractions were supposedly buffer-exchanged into 0.05M Tris-HCl + 1mM EDTA at pH 8 (via a PD-10 Sephadex G-25 column), it was not possible to eliminate the formation of turbidity during enzyme activity assays. This might have been due to the high amount of phosphate present (0.5M PB at pH 8 was used to neutralize the eluted fractions). As a result, 0.05M Tris-HCl at pH 7 and 0.5M Tris-HCl at pH 9 was subsequently used as the starting and neutralizing buffer respectively. Henceforth, frozen HFSN was thawed and buffer-exchanged (PD-10 Sephadex G-25 column) into 0.05M Tris-HCl at pH 7. It was further diluted with 0.05M Tris-HCl at pH 7 to 20-30 mg protein/ml before being loaded onto column. The column was then washed with 0.05M Tris-HCl at pH 7 to remove any unbound or loosely bound proteins. The bound proteins from IAC-I were eluted with 0.05M glycine-HCl at pH 2.3. For IAC-II, 0.05M glycine-HCl at pH 2.8 was introduced followed by 0.10M glycine-HCl at pH 2.3 or ActiSep (Sterogene Bioseparations), a commercial “neutral, nondenaturing” eluent. 0.5M Tris-HCl at pH 9 was used to neutralize the eluted fractions (not required when ActiSep was used). Enzyme activity assay and reducing SDS-PAGE were performed on selected fractions and the starting material.
3.7 Analysis

3.7.1 Protein Concentration Determination

Protein concentration of IgY, BSA and bovine IgG solutions was determined by measuring absorbance at 280 nm and assuming extinction coefficients of 14, 6.8 and 14 cm⁻¹ %⁻¹, respectively (Li-Chan et al., 1998; CRC, 1970). The protein content of fractions during chromatographic procedures were also monitored using absorbance at 280 nm and assuming an extinction coefficient of 10 cm⁻¹ %⁻¹ (Ikura et al., 1987), except for isolation of specific IgY where a value of 14 cm⁻¹ %⁻¹ was used. Where applicable, protein concentration was analyzed by the Biuret microassay using Sigma Diagnostics Total Protein Reagent (Sigma Chemical Co., St. Louis, MO) following the recommended protocol. Alternately, it was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL) or the Bio-Rad Protein Assay kit in the Standard Microassay procedure (Bio-Rad Laboratories Ltd., Mississauga, ON) according to the manufacturers’ recommendations. These three assay methods have different ranges of detection; the choice of kit(s) depends on which of these ranges the predicted protein concentration of a given sample falls within (Table 3.6). BSA was used as the protein standard for the construction of standard curves.
<table>
<thead>
<tr>
<th>Protein Assay Kit</th>
<th>Range of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biuret</td>
<td>1 – 10 mg/ml</td>
</tr>
<tr>
<td>BCA (standard microassay)</td>
<td>0.1 – 1.2 mg/ml</td>
</tr>
<tr>
<td>Bio-Rad</td>
<td>0.0008 – 0.08 mg/ml</td>
</tr>
</tbody>
</table>
3.7.2 Bovine Plasma Factor XIII Activity Assay

3.7.2.1 Principle and Procedure

A monodansylcadaverine-mediated fluorescence activity assay method published by Takagi et al. (1986) was used to measure enzyme activity throughout this work (Figure 3.3).

An important modification was that for each sample assayed a set of controls was also assayed in which with the enzyme activity inhibited by the addition of 1M ammonium sulphate before the first incubation, rather than after the second incubation (as for the sample). Enzyme-catalyzed covalent coupling of monodansylcadaverine (MDC) into some proteins produces a shift in both the wavelength and the intensity of fluorescence of the dansyl group, as if the chromophore were placed in a more hydrophobic environment (Lorand et al., 1971). This forms the basis of the assay method.

All samples and controls were analyzed in singles or duplicates. Controls were identical to samples except for ammonium sulfate, which was added prior to thrombin activation. We have observed the formation of precipitates upon the addition of 0.4 M CaCl₂. The literature has no mention of such phenomenon. We have been using samples containing 0 to 0.3 M NaCl in sodium phosphate buffer. It is therefore possible that the observed precipitate contained calcium phosphate conjugates. Both sonication and vigorous shaking during incubation and just before fluorescence measurements have produced good agreement among duplicates. Sonication was abandoned later due to inconvenience and loss of sample from spills. All this also led to the switch from phosphate buffers to Tris-based buffers in some of the later experiments, to avoid the formation of precipitates. When measuring the enzyme activity of samples containing
phosphate buffer, each sample was first buffer-exchanged into Tris buffer prior to the analysis.
Figure 3.2: Flow-chart of the Monodansylcadaverine-mediated Bovine Plasma FXIII Activity Assay (Takagi et al., 1986)

0.2 ml sample solution +
0.2 ml 20% aq. ethyleneglycol monomethyl ether (cellosolve)
\[ \downarrow \]
Heat\textsuperscript{a} at 56°C for 4 min
\[ \downarrow \]
Cool to r.t. on ice
\[ \downarrow \]
50 μl 0.2 M dithiothreitol in 50% glycerol +
100 μl thrombin in 25 mM Tris-HCl buffer (20 mM CaCl\textsubscript{2}), pH 7.5 +
1.45 ml 0.1 M Borate/KCl buffer with 1.5% cellosolve, pH 9.0 +
50 μl 0.4 M CaCl\textsubscript{2} in 50 mM Tris-HCl, pH 7.5
\[ \downarrow \]
Incubate at 37°C for 20 min
\[ \downarrow \]
50 μl 0.25 mM MDC in 50 mM Tris-HCl, pH 7.5 +
200 μl 5% acetylated casein
\[ \downarrow \]
Incubate at 37°C for 30 min
\[ \downarrow \]
100 μl 1 M ammonium sulphate\textsuperscript{b,c}
\[ \downarrow \]
\[ \lambda_{ex}=350; \lambda_{em}=480 \]

\textsuperscript{a}Thrombin coagulation of fibrinogen (e.g., when assaying plasma) is overcome by a "desensitization" step, involving heating of sample in the presence of ethyleneglycol. This would render the fibrinogen incapable of precipitating. "Desensitization" is not necessary when analyzing purified enzymes.

\textsuperscript{b}Transglutaminase produces ammonia, and an excess amount of ammonium ion should prevent further progress of the reaction. With this technique of stopping, which requires no precipitation and washing to recover substrate, good reproducibility and sensitivity and multiple sample analysis are achieved.

\textsuperscript{c}Control = ammonium sulphate added prior to 20-min incubation
3.7.2.2 Enhancement Factor

According to Takagi et al. (1986), who developed the above assay method, the amount of the incorporated MDC can be expressed in terms of the degree of fluorescence enhancement of the dansyl group after incorporation into protein. This procedure corrects for the variations in the reagents (especially casein) used from one experiment to another.

To do this, a low concentration of MDC was reacted with casein until free MDC is exhausted (second incubation extended from 30 min to 24 hr). The increase in fluorescence intensity was measured, and the "Enhancement Factor" (EF) for the incorporation was defined as the ratio of fluorescence intensity of the protein-incorporated MDC to that of free MDC under the same conditions. It has been previously observed in our laboratory that this increase would have reached its plateau and the fluorescence has maintained itself by 24 hr (data not shown). In the present study, an EF value of 6.0 was determined experimentally, based on the average obtained from two trials.

3.7.2.3 Bovine FXIII Activity Calculations

Our assay method was based on that of Takagi et al. (1986). The following formula was used to derive the bovine FXIII activity:

\[
\frac{\text{MDC}_{\text{incorporated}}}{0.2 \text{ ml sample}} = \frac{(I_f - I_0)}{(E.F. \times I_0) \times \text{MDC}_{\text{total}}}{0.2 \text{ ml sample}} = \frac{\text{n mol MDC}}{30 \text{ min} / 0.2 \text{ ml sample}}
\]
Determination of MDC_total:

(i) 50 µl of a 0.25 mM solution of MDC was added to the reaction mixture, giving rise to a final volume of 2.4 ml.

(ii) Therefore, in terms of the amount of MDC in that mixture:

\[
\text{MDC}_{\text{total}} = 0.25 \text{ mM} \times 50 \mu l
\]
\[
= 0.25 \text{ mmol/L} \times 5 \times 10^{-5} \text{ L}
\]
\[
= 1.25 \times 10^{-5} \text{ mmol}
\]
\[
= 12.5 \text{ nmol}
\]

Sample Calculation of Enzyme Activity:

If \(I_o = 20\), \(I_f = 50\), E.F. = 6, and MDC_total = 12.5 nmol, and by using the following formula:

\[
\text{MDC incorporated}/0.2 \text{ ml sample} = \frac{(I_f - I_o) \times (\text{E.F.} \times I_o) \times \text{MDC total}}{0.2 \text{ ml sample}}
\]
\[
= \text{nmol MDC/30 min/0.2 ml sample}
\]

\[
\text{MDC incorporated}/0.2 \text{ ml sample} = \frac{(50 - 20)}{(6 \times 20)} \times 12.5 \text{ nmol}
\]
\[
= 3.1 \text{ nmol MDC/30 min/0.2 ml sample}
\]

3.7.2.4 Other Considerations

Where \(I_f\) is less than \(I_o\), the activity can not be determined. One unit of activity was defined as one nmol MDC incorporated per 30 min per 0.2 ml sample. Also, the formula given above is for calculating the relative enzyme activity. To determine the specific enzyme activity (nmol MDC/30 min/mg), the value obtained is simply divided
by the protein concentration (mg/ml) in the sample then multiplied by 5. Purification factor is calculated by dividing the specific activity of purified sample by that of starting material.

### 3.7.3 Enzyme-linked Immunosorbent Assay (ELISA)

ELISAs were performed to test the antibody titre of the crude IgY concentrates. The following describes the general procedure used for both sets of ELISAs performed, which is essentially that of Kummer *et al.* (1992): The recipes of the buffers used have been listed in Table 3.7. To each microtiter plate well, 100 µl of PGUB (1 µg/ml) and 100 µl carbonate coating buffer (pH 9.6) were added. The plate was incubated for 1 hr at 37°C, after which it was washed twice with PBS-Tween. The wells were then blocked for 30 min at 37°C, with 250 µl of blotto and washed once with PBS-Tween. At this point, IgY preparations at desired concentrations were applied to the wells (in triplicates) and incubated with the bound antigens for 1 hr at 37°C. The plate was then thoroughly washed thrice with PBS-Tween to remove any unbound IgY. 100 µl of rabbit anti-chicken IgY alkaline phosphatase conjugate (100 ppm) in PBS-Tween was subsequently added and the plate was incubated for an additional hour at 37°C. Afterwards, the plate was washed thrice with PBS-Tween then once with de-ionized distilled water. Finally a substrate of alkaline phosphatase (5 mg alkaline phosphate substrate tablet dissolved in 10 ml diethanolamine buffer at pH 9.8) was added (100 µl per well) and incubated with the conjugated antibody-antigen complex at room temperature for times up to 2 hr. The color development in the wells was measured spectrophotometrically at 405 nm with reference at 690 nm on Labsystems iEMS Reader MF.
In the first set of ELISAs, PGUB were first immobilized onto microtitre plates and bovine plasma FXIII-specific IgY containing crude IgY concentrates were applied to plates and binding was measured.

In the second set of ELISAs, PGUB again were immobilized onto microtitre plates but this time, non-bovine plasma FXIII-specific IgY preparations were also applied in separate wells and binding measured. These IgY preparations were obtained from chicken immunized with cheddar cheese whey and bovine serum IgG.

The IgY concentrate having the highest specific titre was chosen for capturing bovine plasma FXIII. It was noted that PGUB had a very low non-bovine plasma FXIII-specific IgY binding activity.
Table 3.7: Recipes of Buffers Used in ELISA

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Compositions</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonate coating buffer (pH 9.6)</td>
<td>1.59 g Na$_2$CO$_3$ + 2.93 g NaHCO$_3$ + 0.20 g NaN$_3$</td>
<td>Make up to 1 L with distilled water after adjusted pH to 9.6 with HCl.</td>
</tr>
<tr>
<td>Blotto</td>
<td>0.5% skimmilk powder in distilled water (Carnation Canada 1)</td>
<td>Make fresh on the day of assay.</td>
</tr>
<tr>
<td>PBS-Tween</td>
<td>0.05% Tween 20 in PBS [PBS = 8 g NaCl + 0.20g KH$_2$PO$_4$ + 1.15 g Na$_2$HPO$_4$ + 0.20 g KCl + 0.20 g NaN$_3$; make up to 1 L with distilled water after adjusted to pH 7 with HCl]</td>
<td></td>
</tr>
<tr>
<td>Diethanolamine substrate buffer (pH 9.8)</td>
<td>97 ml diethanolamine + 0.10 g MgCl$_2$ + 0.20 g NaN$_3$</td>
<td>Add to 800 ml distilled water and adjust pH to 9.8 with conc. HCl and then bring volume to 1 L.</td>
</tr>
</tbody>
</table>
3.7.4 Reducing SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed on 10-15% PhastGel gradient gels using the PhastSystem electrophoresis unit (Pharmacia Biotech Inc., Baie d'Urfe, PQ). Samples containing 2% SDS, 2% mercaptoethanol, and 0.005% bromophenol blue tracking dye were prepared for electrophoresis by boiling for 10 min. The electrophoresis was performed and the gels were stained with Coomassie brilliant blue according to the manufacturer's recommendations. Where higher sensitivity was required, silver staining was performed following the recommended protocol. As much as possible, protein molecular weight (MW) markers were run along with samples. In particular, SigmaMarkers (High- or Wide-range) were processed and used as recommended for Coomassie Blue staining. For silver-staining, MW marker preparations were diluted to 0.2 to 0.4 mg/ml with 0.05M Tris-HCl at pH 7 (or 0.025M Tris-HCl (1mM EDTA) at pH 8) + 2% SDS + 2% mercaptoethanol prior to boiling. Molecular weight determination of proteins on PhastGels was done with a Pharmacia PhastImage Gel Analyzer.
CHAPTER IV
RESULTS AND DISCUSSION

4.1 Background Results

4.1.1 Starting Materials

Fibrimex used in this work is a frozen bovine fibrinogen preparation that can be applied for the binding of meat according to a patented process. Fibrin (derived from fibrinogen) is the major protein that makes up the blood clots. The thrombin used as one of the reagents in the enzyme activity assay induces clotting of fibrinogen (Lorand et al., 1971). When Fibrimex (or other blood products containing fibrinogen) is used as the starting material from which bovine plasma FXIII is derived, fibrinogen may be readily removed by its differential denaturation via heating (Loewy et al., 1957; also described in Section 3.4.1). This heating step was repeated during my research several times from essentially two batches of Fibrimex. The average yield of protein in the supernatants obtained from the two batches after heating (HFSN) were 31 ± 19 (n=5) and 35 ± 3.2 (n=3) % total protein. The specific activity of the second batch of Fibrimex before heat treatment was estimated to be 2.6 units/ mg total protein.

4.1.2 Enzyme Activity Assays

The procedure used herein to determine plasma FXIII enzyme activity was essentially that of Takagi et al. (1986). The amount of 0.1 M borate buffer, however, was increased from 1.20 ml to 1.45 ml (final reaction volume = 2.4 ml).
Fluorescence intensity (indicative of the enzyme activity present) has been shown in the literature to increase linearly with reaction time up to 30 min (Takagi et al., 1986). Also, during the early stage of this work precipitation in the reaction mixture was observed when phosphate-based buffers were used (e.g., during ion-exchange chromatography). Since precipitation happened before the addition of acetylated casein reagent, the formation of insoluble calcium phosphate salts may be implicated. As a result, Tris buffers were subsequently used instead where applicable. An enhancement factor of 6.0 was obtained in this work. This value is comparable to that obtained in our laboratory previously by Karoline Lee (i.e., 6.53) but is only half of what was reported for fresh bovine plasma (i.e., 13) by Takagi et al. (1986).

To determine the range of protein concentrations within which a linear relationship between enzyme activity and the actual amount of enzymes is maintained, the dilution linearity of enzyme activity under our assay conditions was investigated. Using heat-treated Fibrinex (HFSN) and purified bovine plasma FXIII obtained via immunoffinity chromatography, linearity was obtained over these ranges studied: 0.21 – 3.4 and 0.055 – 0.22 mg/ml respectively (Figures 4.1A and 4.1B).

The average specific activity of HFSN prepared over a period of two years was determined to be 4.37 ± 2.13 units/ mg protein (n=12). The values obtained in individual assays varied from 1.01 to 8.30 units/ mg protein. 20% saturated ammonium sulphate precipitation of Fibrinex was also performed prior to heating at 56°C for 4 min. The specific activity of the HFSN obtained was 30.3 units/ mg protein which was several fold greater than that of HFSN prepared without ammonium sulphate precipitation. However, this alternative route was not pursued, after subsequent ion-exchange chromatography
failed to produce fractionation of enzyme activity under the conditions generally used throughout this work (data not shown).
Figure 4.1A: Dilution Linearity of Enzyme Activity Assay of Heat-treated Fibrimex (HFSN)

Linear regression equation: $Y = 0.811 \times X + 0.0804 \quad (R^2 = 0.993)$

[a] Linear regression equation: $Y = 0.811 \times X + 0.0804 \quad (R^2 = 0.993)$

[b] [Protein] was determined using Biuret microassay as described in Section 3.7.1.
Figure 4.1B: Dilution Linearity of Enzyme Activity Assay of Bovine Plasma FXIII Purified via Immunoaffinity Chromatography

Relative Activity (units/0.2 ml sample) vs. Assay [Protein] (mg/ml)

- Relative Activity
- Linear (Relative Activity)

Linear regression equation: \( Y = 3.06 \times X + 0.0223 \) (\( R^2 = 0.996 \))

\( ^a \)Linear regression equation: \( Y = 3.06 \times X + 0.0223 \) (\( R^2 = 0.996 \))

\( ^b \) [Protein] was determined using the BCA protein assay kit as described in Section 3.7.1.
4.2 Preliminary Work on Ion-exchange Chromatography

If one assumes that the isoelectric point for bovine plasma FXIII follows the same trend as its human counterpart, bovine plasma FXIII would carry a net negative charge at pH above 5.2 (McDonagh, 1994) and also near its physiological pH where the enzyme is likely to be most stable. In this state, the protein has the potential to bind to anion-exchangers, which should effect its purification. Two kinds of ion-exchange resins were employed in this work. The first, DEAE Sepharose CL-6B is a macroporous bead-formed weak ion-exchanger derived from the crosslinked agarose gel Sepharose CL-6B. In general, it has good chemical and physical stability as well as flow properties. However, prolonged exposure to very alkaline conditions should be avoided because of the inherent instability of the DEAE group as a free base. The second kind of resin, RESOURCE Q, is a pre-packed strong ion-exchange column based on rigid, monodisperse micro-beads made of polystyrene/divinyl benzene.

The binding of bovine plasma FXIII to the anion exchangers was demonstrated by the concentration of enzyme activity in the eluted fractions (Table 4.1). RESOURCE Q ion-exchange column, connected to the Pharmacia Biotech’s FPLC system, was used to elucidate the range of ionic strength necessary to elute the enzyme. The results obtained were then used to help establish the conditions for larger scale low-pressure chromatography with DEAE Sepharose CL-6B resin. The elution of enzyme activity was mostly restricted to between 0.088 and 0.12M NaCl (e.g., conductivity of ~ 0.07 mS) in the eluting buffer (Table 4.1). While this is in agreement with previous work done at this Department, it is higher than the value given in the literature (around 0.05M NaCl) for a comparable method (Lorand et al., 1968).
The average increase in specific activity achieved by preliminary chromatography experiments using RESOURCE Q and DEAE-Sepharose CL-6B were 10.8 ± 7.34 (n=3) and 4.4 ± 2.5 (n=3) respectively (Table 4.1). Continuous gradient was difficult to produce on the low-pressure system and therefore, only step-wise gradient elution was used. Figures 4.2A and 4.3A show the conditions and results obtained from typical ion-exchange chromatography experiments using the two resins. When selected fractions from these experiments were analyzed using reducing SDS-PAGE (Figure 4.2B and 4.3B), it became apparent that one of the most prominent proteins present in the enzyme rich fractions was bovine IgG (separated into heavy- (IgG-HC) and light-chains (IgG-LC) upon electrophoresis; Lane 4 and 5 in both figures). Also, a considerable amount of serum albumin (BSA) was fractionated by virtue of being eluted at a higher ionic strength (at conductivity near 5 mS during FPLC) than bovine plasma FXIII (Lane 2 in Figure 4.2B and Lane 3 in Figure 4.3B). Results obtained by PhastImage gel analyzer seem to confirm these observations, that is, the presence of IgG-HC, IgG-LC and BSA in the lanes noted (APPENDIX II).

The bands corresponding to bovine plasma FXIII (Lane 1, 4 and 5 in Figure 4.2B and Lane 4 and 5 in Figure 4.3B), if visible, are not readily identifiable. The $a$ and $b$ subunits that make up the bovine plasma FXIII tetramer ($a_2b_2$) are held together by strong, yet noncovalent interactions (McDonagh, 1994). Therefore, SDS alone should dissociate the subunits from each other. In the presence of mercaptoethanol (as in reducing SDS-PAGE), the $a$ and $b$ subunits tend to co-migrate very closely (McDonagh, et al., 1976; McDonagh, 1994). Bovine plasma FXIII purified by Ikura et al. (1987) was shown to separate into two distinct bands upon non-reducing SDS-PAGE. Visual
inspection of the illustration as it appears in the publication seems to suggest an apparent molecular weight of ~80 and ~67 kDa for the \(a\) and \(b\) subunits respectively. These values are lower than those generally reported for human plasma FXIII, for example, between 82 and 83 kDa for \(a\) subunits and around 76 kDa for \(b\) subunits (McDonagh, 1994). The glycosylated nature of the \(b\) subunits may also affect the mobility of these subunits upon electrophoresis. Based on this knowledge, the bands corresponding to an apparent molecular weight of ~76 and ~70 kDa (Lane 4 and 5 in Figure 4.2B; see APPENDIX II) may possibly be those of the \(a\) and \(b\) subunits respectively. Similar bands also appear in Figure 4.3B (Lane 4 and 5; see APPENDIX II) but were too weak to be detected by the gel analyzer. It is not clear why these bands also appeared in Lane 6 which corresponds to a sample having almost no enzyme activity. It remains possible that the band(s) corresponding to bovine plasma FXIII and its subunits are in fact not visible due to their low concentration relative to the other proteins in the sample.

Yields of greater than 100% were obtained from two of the experiments using RESOURCE Q resins. These might have been due to the low specific activities of the starting materials as well as error in the estimated protein concentration of the fraction bearing the highest specific activity.
"Absorbance peaks are major peaks observed on the chromatograms."

"% Yield: % total activity (specific activity x total protein) at the peak indicated relative to the total activity of starting material loaded."

"Protein was determined using the direct microassay as described in Section 3.1."multipled by a (UV) monitor optical cell path length = 2 cm, assuming an extinction coefficient of 1.00 cm⁻¹."

"Protein was estimated by adding absorbance at 280 nm (corresponding to the fraction) then divided by the number of readings, and calculated by multiplying by a fraction concentration of 1.0 M."
Figure 4.2.4: Ion-exchange Chromatographic Partition of Bovine Plasma FXIII on RESOURCE Q Column

Chromatogram:

Fraction size: 1 ml
Flow rate: 1 ml/min, sample loaded at 0.5 ml/min
Buffer: 25 mM Tris- HCl (1 mM EDTA) at pH 8.0
Running buffer: 0.25 M NaCl followed by 0.5 M NaCl in starting buffer
Eluting buffer: Continuous gradient 0-1 M NaCl in starting buffer
Sample: 1 ml (pre-packed by Pharmacia Biotech Inc.)
Resin: RESOURCE Q, 1 ml (pre-packed by Pharmacia Biotech Inc.)
Temperature: 4°C

Absorbance at 280 nm — Conductivity (mS)

Absorbance at 280 nm

Conductivity (mS)
Figure 4.2B: Reducing SDS-PAGE of Selected Fractions from Ion-exchange Chromatographic Purification of Bovine Plasma FXIII on RESOURCE Q Column (See Figure 4.2A)

Lane¹ 1 2 3 4 5 6 7

Possible bovine plasma FXIII bands

Lane 1  Starting material
Lane 2  Fraction 22
Lane 3  Fraction 18
Lane 4  Fraction 15
Lane 5  Fraction 14
Lane 6  Fraction 3
Lane 7  SigmaMarkers (High-Range)

¹Lane 1 Starting material
Lane 2 Fraction 22
Lane 3 Fraction 18
Lane 4 Fraction 15
Lane 5 Fraction 14
Lane 6 Fraction 3
Lane 7 SigmaMarkers (High-Range)

²Gel stained with Coomassie Brilliant Blue.
Figure 4.3A: Ion-exchange Chromatographic Purification of Bovine Plasma FXIII on DEAE-Sepharose CL-6B Resin

Chromatography conditions:
Temperature: Room temperature
Resin: DEAE-Sepharose CL-6B (Pharmacia Biotech Inc.)
Bed dimensions: 2.5 cm (d) x 8.5 cm (h)
Packing flow rate: 100 ml/hr
Running flow rate: 60 ml/hr
Starting buffer: 25mM Tris-HCl (1mM EDTA) at pH 8.0
Elution buffers: 0.04, 0.10, and 1M NaCl in starting buffer
Starting material: HFSN (30 ml at 3.7 mg/ml)
Fraction size: 6 ml

Chromatogram:
Figure 4.3B: Reducing SDS-PAGE of Selected Fractions from Ion-exchange Chromatographic Purification of Bovine Plasma FXIII on DEAE-Sepharose CL-6B (See Figure 4.3A)

Lane 1 = SigmaMarkers (Wide-Range)
Lane 2 = Starting material
Lane 3 = Fraction 80
Lane 4 = Fraction 56
Lane 5 = Fraction 55
Lane 6 = Fraction 37
Lane 7 = Bovine serum IgG
Lane 8 = Bovine serum IgG

Possible bovine plasma FXIII bands

a Lane 1 = SigmaMarkers (Wide-Range)
Lane 2 = Starting material
Lane 3 = Fraction 80
Lane 4 = Fraction 56
Lane 5 = Fraction 55
Lane 6 = Fraction 37
Lane 7 = Bovine serum IgG
Lane 8 = Bovine serum IgG

b Gel stained with Coomassie Brilliant Blue.
4.3 Bovine Plasma FXIII Purification for Immunoaffinity Column Preparation

In view of the presence of bovine immunoglobulins in the enzyme rich fractions obtained from ion-exchange experiments, it was decided that such preparation should be subjected to Protein G affinity chromatography. The ligands linked to this affinity resin bind specifically and strongly to immunoglobulins from a variety of sources, including those of the bovine origin. Preliminary experiment using ImmunoPure (G) Immobilized Protein G (Pierce) column demonstrated the effectiveness of this technique in containing the bovine immunoglobulins in the eluted fractions (see Figures 4.4A and 4.4B). In particular, note the resemblance in appearance between the eluted fraction (Lane 2) and a commercial bovine IgG (Lane 6) upon electrophoresis. This was confirmed by results from PhastImage gel analyzer (see APPENDIX II). A purification factor of 4.31 and a yield of 71.9% were obtained during this step. Enzyme activity was restricted to the unbound and wash fractions. However, band(s) corresponding to bovine plasma FXIII and its subunits were not visible upon electrophoresis due to low protein concentration in the sample (Lane 3 and 4).

By coupling low-pressure ion exchange and Protein G affinity chromatography, a protein preparation rich in bovine plasma FXIII was produced as follows. Enzyme rich fractions from an ion-exchange experiment (Figure 4.5 and Table 4.2) were pooled and subjected to Protein G affinity chromatography (Figures 4.6 and Table 4.3) in six runs under similar conditions using Protein G HiTrap affinity column (Pharmacia Biotech). The capacity of this column was estimated to be about 20 mg. A purification factor and a yield of 3.0 and 89% respectively were achieved in the latter step for one of the runs. The
bovine plasma FXIII rich preparation (PGUB) thus obtained was purified 7.13 fold with respect to the enzyme than the ion-exchange starting material (HFSN).

In the subsequent experiment, PGUB (22 mg protein) was immobilized to construct the immunoaffinity column to be used for the isolation of specific yolk immunoglobulins against bovine plasma FXIII. Half of the sample (11 mg) was dialyzed into 0.1M PB at pH 7 and used for coupling onto the Actigel ALD Superflow resin (potential degree of substitution up to 50-60 mg ligand/ ml resin). However, little binding (8.7%) was obtained. This was probably due not to the overloading of resins but rather the inadequacy of dialysis in preventing the presence of trace amount of Tris salts, which interfere with the coupling reaction. Buffer-exchange of the sample via a PD-10 Sephadex G-25 column prior to incubation with coupling reagent improved ligand binding approximately five times, to 44% (Table 4.4). Also, the resulting degree of substitution (1.6 mg ligand/ ml resin) fell within the manufacturer’s recommendations for enzyme immobilization (1-5 mg ligand/ ml resin).

The resulting column which contains immobilized bovine plasma FXIII is denoted hereinafter by FC ("FXIII Column").
Figure 4.4A: Protein G Affinity Chromatography of an Enzyme Rich Fraction from Ion-exchange Chromatography

Chromatography conditions:
Temperature: Room temperature
Resin: ImmunoPure (G) Immobilized Protein G Gel (Pierce)
Bed dimensions: 0.5 ml (vol)
Running flow rate: 2 ml/hr
Binding buffer: 0.2M acetate buffer at pH 5.0
Elution buffer: 0.10M Glycine-HCl at pH 2.7
Neutralizing buffer: 1M Tris-HCl at pH 9.0
Starting material: An enzyme rich fraction from previous ion-exchange chromatography (2 ml at 1.2 mg/ml)
Fraction size: 1 ml

Chromatogram:
Figure 4.4B: Reducing SDS-PAGE of Selected Fractions from Protein G Affinity Chromatography (See Figure 4.4A)

Lane$^a$ 1 2 3 4 5 6 7

kDa
116
66
29
6.5

Lane 1 = HFSN
Lane 2 = Fraction 8
Lane 3 = Fraction 3
Lane 4 = Fraction 2
Lane 5 = Starting material
Lane 6 = Bovine serum IgG
Lane 7 = SigmaMarkers (Wide-Range)

$^a$Lane 1= HFSN
$^b$Gel stained with Coomassie Brilliant Blue.
**Figure 4.5:** Ion-exchange Chromatographic Purification of Bovine Plasma FXIII

**Chromatogram:**

- Fraction size: 6 ml (12 ml for sample loading and wash)
- Eluting buffer: 0.04M, 0.12M & 0.1M NaCl in starting buffer
- Starting buffer: 25mM Tris-HCl (1mM EDTA, pH 8)
- Starting material: HPSN (120 ml at 3.56 ug/ml)
- Operational flow rate: 1 ml/min
- Packing flow rate: 1.8 ml/min
- Bed dimensions: 2.5 cm (d) x 9.8 cm (h); 48 ml (vol)
- Resin: DEAE-Sepharose CL-6B
- Temperature: Room temperature

Absorbance at 280 nm
<table>
<thead>
<tr>
<th>Protein (Volume)</th>
<th>Protein (Specific activity)</th>
<th>Protein (Total activity)</th>
<th>Protein (Specific activity)</th>
<th>Protein (Total activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.46</td>
<td>96</td>
<td>0.6</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>1.2</td>
<td>20</td>
<td>0.2</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>49</td>
<td>22</td>
</tr>
<tr>
<td>1.49</td>
<td>2.2</td>
<td>0.32</td>
<td>120</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Table 4.2: Ion-exchange Chromatographic Fractionation of Bovine Plasma FXIII
Figure 4.6: Protein G Affinity Chromatography of Enzyme Rich Fractions from Ion-exchange Chromatography

**Chromatography conditions:**
- **Temperature:** Room temperature
- **Resin:** HiTrap Protein G (Pharmacia Biotech Inc)
- **Bed dimensions:** 1 ml (vol)
- **Packing flow rate:** Manufacturer
- **Operating flow rate:** 1 ml/min
- **Starting material:** Enzyme activity rich fraction from previous ion exchange experiment (5 ml at 2.22 mg/ml)
- **Starting buffer:** 25mM Tris-HCl (1mM EDTA) at pH 7
- **Eluting buffer:** 0.1M Glycine-HCl at pH 2.7
- **Neutralizing buffer:** 1M Tris-HCl at pH 9 (0.05 ml column effluent + 0.05 ml neutralizing buffer)
- **Fraction size:** 1 ml

**Chromatogram:**

![Chromatogram](image)
Purification factor over starting material = 3.1 fold; purification factor over HPFN = 7.13 fold

Purification factor over starting material. % Yield = Total activity against initial of the starting material

Specific activity = Total protein * Specific activity

A total of six chromatography runs were done: Data shown were averages of run no. 1 and 6.

<table>
<thead>
<tr>
<th>Eluted (fraction 14-17) µl</th>
<th>Unbound (fraction 4-10) µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0370 0.480 10.5 0.0379 2.62 0.00</td>
<td>7.00 0.481 3.41 1.52</td>
</tr>
<tr>
<td>8.89 11.31 3.37 11.2 2.24 0.00</td>
<td></td>
</tr>
<tr>
<td>100 1.295 11.5 5.00</td>
<td></td>
</tr>
</tbody>
</table>

Starting material

<table>
<thead>
<tr>
<th>(ml)</th>
<th>(µl)</th>
<th>(units)</th>
<th>(units/ml)</th>
<th>(µg/ml)</th>
<th>(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(units)</td>
<td>Protein activity</td>
<td>Protein activity</td>
<td>Pool</td>
<td>Total</td>
<td>Specific activity</td>
</tr>
<tr>
<td>(units/ml)</td>
<td>Protein activity</td>
<td>Protein activity</td>
<td>Pool</td>
<td>Total</td>
<td>Specific activity</td>
</tr>
<tr>
<td>(µg/ml)</td>
<td>Pool</td>
<td>Total</td>
<td>Specific activity</td>
<td>Pool</td>
<td>Total</td>
</tr>
<tr>
<td>(µg/ml)</td>
<td>Pool</td>
<td>Total</td>
<td>Specific activity</td>
<td>Pool</td>
<td>Total</td>
</tr>
</tbody>
</table>

Ion-exchange Chromatography (see Figure 4.6)

Table 4.3: Protein Affinity Chromatography of Enzyme Rich Fractions from

(see image for more detailed content)
Table 4.4: Immobilization of Bovine Plasma FXIII Rich Fractions onto Actigel ALD-Superflow (FC)\(^a\)

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Absorbance at 280 nm</th>
<th>[Protein] (mg/ml)(^b)</th>
<th>Total Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>7.00</td>
<td>1.590</td>
<td>1.59</td>
</tr>
</tbody>
</table>

Wash schedule after immobilization:

<table>
<thead>
<tr>
<th>Effluent during packing</th>
<th>7.5</th>
<th>0.72</th>
<th>0.72</th>
<th>5.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M PB at pH 7</td>
<td>5.0</td>
<td>0.14</td>
<td>0.14</td>
<td>0.72</td>
</tr>
<tr>
<td>0.5M NaCl PBS at pH 7</td>
<td>5.0</td>
<td>0.0040</td>
<td>0.0040</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.0030</td>
<td>0.0030</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.0030</td>
<td>0.0030</td>
<td>0.015</td>
</tr>
<tr>
<td>0.05M glycine-HCl at pH 2.8</td>
<td>5.0</td>
<td>0.012</td>
<td>0.012</td>
<td>0.060</td>
</tr>
</tbody>
</table>

Mass balance (mg):

| Starting material | 11.1 |
| Total Unbound     | 6.2  |
| Bound             | 4.9  |
| % Binding         | 44%  |

\(^a\)Bed volume = 3 ml

\(^b\)Extinction coefficient = 10 cm\(^{-1}\) %\(^{-1}\) (Ikura et al., 1987)
4.4 Specific IgY Purification

4.4.1 Crude IgY and ELISA

Crude IgY preparations were prepared from eggs laid by hens previously immunized with bovine plasma FXIII prepared by Karoline Lee and Angela Gerber (Department of Food Science, UBC) and stored frozen until use.

ELISA assays were done to assess the ability of these crude IgY preparations to bind to the bovine plasma FXIII rich preparation (PGUB). Three dilutions of crude IgY protein (concentrations of 2, 1 and 0.1 μg/ml) were allowed to react with PGUB immobilized onto a microtiter plate. The enzyme-linked assays produced a color whose absorbance was measured. The plate was read after 1 hour of incubation and again at 2 hour. The preparation from which the highest absorbances (highest activity) were obtained was chosen as the source from which specific IgY were isolated. To differentiate between specific and nonspecific binding, a second set of ELISA assays (See Table 4.5) was performed. The results are shown in Table 4.6.
<table>
<thead>
<tr>
<th>Crude IgY</th>
<th>Protein concentration used in ELISA (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(obtained from eggs laid by hens immunized with the given protein)</td>
<td></td>
</tr>
</tbody>
</table>

Specific:\(^a\):
- Bovine plasma FXIII (LEE\(^b\))

Nonspecific:\(^a\):
- Cheddar cheese whey IgG: 1000, 100, 10, 2
- Human IgG: 1000, 100, 10, 2

\(^a\)The adjectives Specific and Nonspecific refer to the immunization of hens with the target antigens (i.e., bovine plasma FXIII) or with other proteins respectively. The former may contain specific IgY against bovine plasma FXIII whereas the latter should not.

\(^b\)LEE = “Lee’s Enzyme Extract” (see Section 3.3.1)
Table 4.6: Results of ELISA (Specific<sup>a</sup> versus Non-Specific<sup>b</sup>)

<table>
<thead>
<tr>
<th>Crude IgY (obtained from eggs laid by hens immunized with the given protein)</th>
<th>[IgY] (μg/ml)</th>
<th>Average absorbance at 405 nm (30 min incubation)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Standard deviation&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine plasma FXIII</td>
<td>10</td>
<td>2.091</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.534</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.851</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.526</td>
<td>0.009</td>
</tr>
<tr>
<td>Non-specific:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheddar cheese whey IgG</td>
<td>1000</td>
<td>1.720</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.525</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.149</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.044</td>
<td>0.003</td>
</tr>
<tr>
<td>Human IgG</td>
<td>1000</td>
<td>2.259</td>
<td>0.074</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.795</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.178</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.054</td>
<td>0.013</td>
</tr>
</tbody>
</table>

<sup>a</sup>See footnotes of Table 4.5.

<sup>b</sup>Four replicate wells (n=4) except for Cheddar cheese whey IgG (n=3)
As evident in Table 4.6, PGUB bound to specific crude IgY to a much greater extent than it did nonspecific IgY. For instance, at the IgY concentration of 2 µg/ml, the absorbances obtained from the specific crude IgY averaged around 15-20 times those from the nonspecific samples. Also, even at the highest concentration (1000 µg/ml), the averaged absorbances obtained from the nonspecific crude IgY were still only comparable with or even lower than those from the specific samples at a much lower concentration (10 µg/ml).

4.4.2 Purification of Specific IgY

4.4.2.1 One-step Method

Bovine plasma FXIII-specific IgY was isolated in one step using the column containing the enzyme (FC; see Section 4.3). The yield of specific IgY appeared to be quite reproducible (Table 4.7). Fig 4.7 illustrates the conditions typically used and the results obtained. The specific IgY (SIGY1) was eluted by 0.05M glycine-HCl at pH 2.8. Assuming the molecular weights of bovine plasma FXIII and IgY are 320 and 180 kDa respectively and that IgY has a valence of 2 (McDonagh, 1994, for human plasma FXIII; Akita and Li-Chan, 1997), the theoretical amount of specific IgY obtainable by FC (4.9 mg immobilized bovine plasma FXIII/ 3 ml resin) would be 1.38 mg. Hence, the average experimental amount of specific IgY obtained, at 1.0 ± 0.18 mg (n=13) per run (Table 4.7), represents ~70% of the theoretical maximum.

The yield (specific over total crude IgY) of bovine plasma FXIII-specific IgY obtained using the one-step method was 4.1 ± 0.43 % (n=13). This is about half of the yield of anti-lactoferrin IgY obtained by Li-Chan et al. (1998) by means of an
immobilized lactoferrin column (~10%). Since the capacity of FC was not known, 4.1% may represent a minimum (if column overloaded) or a maximum (if column underloaded) value obtainable. The immobilization of SIGY1 resulted in 77% binding (Table 4.8) The resulting immunoaffinity column (IAC-I) contained 4.2 mg bound IgY (3 ml Actigel ALD-Superflow resins).

While the details of the results obtained using IAC-I for the purification of bovine plasma FXIII will be discussed in a later section, it suffices to say that reducing SDS-PAGE revealed the presence of bovine serum albumin (BSA), among other proteins, in the eluted fractions. It was hypothesized that a portion of the specific IgY on IAC-I column was in fact cross-reactive with BSA. Consequently, it was decided that an additional adsorption step be introduced during specific IgY purification in an effort to remove those antibodies (subtractive immunoaffinity chromatography).
Table 4.7: Immunoaffinity Purification of Specific IgY (One-Step Method)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Amount of crude IgY applied (mg)\textsuperscript{c}</th>
<th>Amount of specific IgY eluted (mg)\textsuperscript{b,c}</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>1.2</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>0.61</td>
<td>4.1</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>1.0</td>
<td>3.9</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>0.73</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>0.99</td>
<td>3.7</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>1.1</td>
<td>4.3</td>
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<tr>
<td>7</td>
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<td>1.0</td>
<td>3.8</td>
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<tr>
<td>8</td>
<td>27</td>
<td>1.1</td>
<td>4.0</td>
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<td>9</td>
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<td>4.5</td>
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<td>4.3</td>
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<td>11</td>
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<td>1.2</td>
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<td>12</td>
<td>26</td>
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<td>4.4</td>
</tr>
<tr>
<td>13</td>
<td>26</td>
<td>1.1</td>
<td>4.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}3-ml column (4.9 mg PGUB/3 ml Actigel ALD-Superflow resins)

\textsuperscript{b}Amount of Specific IgY = (total absorbance of eluted fractions \times fraction size) / 1.4

\textsuperscript{c}Extinction coefficient of IgY = 14 cm\textsuperscript{-1}%\textsuperscript{-1}. 
**Figure 4.7: Affinity Chromatographic Purification of Bovine Plasma FXIII-Specific IgY (One-step Method)**

**Chromatography conditions:**
- **Temperature:** Room temperature
- **Resin:** Actigel ALD-Superflow (4.9 mg antigens$^a$ / 3 ml resins)
- **Bed dimensions:** 3 ml (vol)
- **Packing flow rate:** Gravity
- **Operating flow rates:** Sample loading = 0.2 ml/min, Wash = 0.73 ml/min, Elution = 0.36 ml/min
- **Starting material:** Crude IgY (18 ml at 1.438 mg/ml)
- **Starting buffer:** 0.01M PB at pH 7
- **Eluting buffer:** 0.05M Glycine-HCl at pH 2.8
- **Neutralizing buffer:** 0.5M PB at pH 8 (0.9 ml column effluent + 0.1 ml neutralizing buffer)
- **Fraction size:** 1 ml (1.1 ml for wash)

**Chromatogram:**

$^a$Enzyme rich fractions obtained from Protein G affinity chromatography (See Figure 4.6)
### Table 4.8: Immobilization of Specific IgY onto Actigel ALD-Superflow\(^a\) (IAC-I)

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Absorbance at 280 nm</th>
<th>[Protein] (mg/ml)(^b)</th>
<th>Total Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material (SIGY1)</td>
<td>21</td>
<td>0.36</td>
<td>0.26</td>
<td>5.4</td>
</tr>
<tr>
<td>Wash schedule after immobilization:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effluent during packing</td>
<td>23</td>
<td>0.060</td>
<td>0.043</td>
<td>0.96</td>
</tr>
<tr>
<td>0.1M PB at pH 7</td>
<td>5.0</td>
<td>0.025</td>
<td>0.018</td>
<td>0.089</td>
</tr>
<tr>
<td>0.5M NaCl PBS at pH 7</td>
<td>5.0</td>
<td>0.0020</td>
<td>0.0014</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.0050</td>
<td>0.0036</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.0040</td>
<td>0.0029</td>
<td>0.014</td>
</tr>
<tr>
<td>0.05M glycine-HCl at pH 2.8</td>
<td>5.0</td>
<td>0.034</td>
<td>0.024</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Mass balance (mg):
- Starting material: 5.4
- Total Unbound: 1.2
- Bound: 4.2
- % Binding: 78%

\(^a\)Bed volume = 3 ml

\(^b\)Extinction coefficient = 14 cm\(^{-1}\)\%/l
4.4.2.2 Two-step Method

The yield of eluted IgY appeared to be reasonably reproducible (Table 4.11) with respect to both subtractive immunoaffinity chromatography and the subsequent isolation of specific IgY using FC. The average amount and yield of IgY cross-reactive with BSA were $2.18 \pm 0.61$ and $2.91 \pm 0.42 \%$ respectively (n=9). The average amount and yield of bovine plasma FXIII-specific IgY obtained in the second step (FC) were $0.64 \pm 0.11 \text{ mg}$ and $1.69 \pm 0.25 \%$ respectively (n=14). The latter values were lower than those obtained with one-step method, due to the removal of BSA cross-reactive IgY that would have contributed to the total amount of bovine plasma FXIII-specific IgY obtainable. Again, the average yield of 1.69% may present a minimum (if column overloaded) or a maximum (if column underloaded) value obtainable. It has been reported that the amount of specific IgY relative to that of crude IgY ranged from 5% (anti-human insulin IgY) to 28% (anti-mouse-IgG IgY) (Hatta et al., 1997).

A drop in the yield was observed after repeated use of both columns. This might be due to specific or nonspecific binding of IgY that was resistant to the eluting conditions applied. Figures 4.8 and 4.9 illustrate the conditions typically used and the results obtained for each of these procedures. Some of the unbound IgY fractions from the second step were pooled and passed through the same column (FC) again; residual binding was estimated to be 0.3% of the IgY applied (i.e., about one-third of the amount obtained in the first pass). The immobilization of specific IgY (SIGY2) thus obtained resulted in 80% binding (Table 4.12). The resulting immunoaffinity column (IAC-II) contained 2.1 mg bound IgY (2 ml resin).
### Table 4.9: Immunoaffinity Purification of Specific IgY (Two-step Method)

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Amount of crude IgY applied (mg)</th>
<th>Amount of specific IgY eluted (mg)</th>
<th>Yield (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 1&lt;sup&gt;a&lt;/sup&gt;</strong> (Subtractive immunoaffinity chromatography):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>60.8</td>
<td>1.99</td>
<td>3.27</td>
</tr>
<tr>
<td>2</td>
<td>91.1</td>
<td>3.23</td>
<td>3.55</td>
</tr>
<tr>
<td>3</td>
<td>69.4</td>
<td>2.08</td>
<td>3.00</td>
</tr>
<tr>
<td>4</td>
<td>69.4</td>
<td>2.23</td>
<td>3.21</td>
</tr>
<tr>
<td>5</td>
<td>42.0</td>
<td>1.17</td>
<td>2.79</td>
</tr>
<tr>
<td>6</td>
<td>114.3</td>
<td>2.76</td>
<td>2.41</td>
</tr>
<tr>
<td>7</td>
<td>82.8</td>
<td>2.04</td>
<td>2.46</td>
</tr>
<tr>
<td>8</td>
<td>74.8</td>
<td>1.91</td>
<td>2.55</td>
</tr>
<tr>
<td><strong>Step 2&lt;sup&gt;a&lt;/sup&gt;</strong> (Isolation of specific IgY using immobilized bovine plasma FXIII (FC)):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28.2</td>
<td>0.684</td>
<td>2.42</td>
</tr>
<tr>
<td>2</td>
<td>28.2</td>
<td>0.526</td>
<td>1.86</td>
</tr>
<tr>
<td>3</td>
<td>27.4</td>
<td>0.457</td>
<td>1.57</td>
</tr>
<tr>
<td>4</td>
<td>41.2</td>
<td>0.717</td>
<td>1.74</td>
</tr>
<tr>
<td>5</td>
<td>41.2</td>
<td>0.721</td>
<td>1.75</td>
</tr>
<tr>
<td>6</td>
<td>44.6</td>
<td>0.697</td>
<td>1.56</td>
</tr>
<tr>
<td>7</td>
<td>41.5</td>
<td>0.650</td>
<td>1.57</td>
</tr>
<tr>
<td>8</td>
<td>37.8</td>
<td>0.599</td>
<td>1.59</td>
</tr>
<tr>
<td>9</td>
<td>41.8</td>
<td>0.760</td>
<td>1.82</td>
</tr>
<tr>
<td>10</td>
<td>41.8</td>
<td>0.597</td>
<td>1.43</td>
</tr>
<tr>
<td>11</td>
<td>43.9</td>
<td>0.623</td>
<td>1.42</td>
</tr>
<tr>
<td>12</td>
<td>43.9</td>
<td>0.764</td>
<td>1.74</td>
</tr>
<tr>
<td>13</td>
<td>43.9</td>
<td>0.747</td>
<td>1.70</td>
</tr>
<tr>
<td>14</td>
<td>27.9</td>
<td>0.413</td>
<td>1.48</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pairs of runs of Step 1 were done (no. 1 and 2, no. 3 and 4, no. 5 and 6, no. 7 and 8). After each set, the unbound fractions (containing bovine plasma FXIII-specific IgY) were pooled and each pool was applied onto FC in 3 to 4 similar runs in Step 2. Specifically, the following sets of Step 2 were performed: set 1 (no. 1-3), set 2 (no. 4-6), set 3 (no. 7-10), set 4 (no. 11-14).

<sup>b</sup>Amount of Specific IgY = (total absorbance of eluted fractions * fraction size) / 1.4

<sup>c</sup>Extinction coefficient of IgY = 14 cm<sup>-1</sup> %<sup>-1</sup>

<sup>d</sup>Yield (%): Step 1 = IgY cross-reactive with BSA; Step 2 = bovine plasma FXIII-specific IgY
Figure 4.8: Immunoaffinity Purification of Bovine Plasma FXIII-Specific IgY (Subtractive Immunoaffinity Chromatography)

Chromatography conditions:
Temperature: Room temperature
Resin: Actigel ALD-Superflow (15.8 mg BSA/5 ml resins)
Bed dimensions: 5 ml (vol)
Packing flow rate: Gravity
Operating flow rates: Sample loading = 0.225 ml/min, Wash = 1 ml/min, Elution = 0.45 ml/min
Starting material: Crude IgY (60.75 mg at 13.5 mg/ml)
Starting buffer: 0.01M PB at pH 7
Eluting buffer: 0.05M Glycine-HCl at pH 2.8, 0.10M Glycine-HCl at pH 2.3\(^b\)
Neutralizing buffer: 0.5M PB at pH 8 (1 part neutralizing buffer per 9 parts column effluent for pH 2.8 elution); 0.5M Tris-HCl at pH 9 (12 parts neutralizing buffer per 88 parts column effluent for pH 2.3 elution)
Fraction size: 2 ml

Chromatogram\(^a\):

\(^a\)Only elution was shown. Typically, 2 - 3 similar runs were performed. The unbound fractions were pooled, from which bovine plasma factor XIII-specific IgY were collected.

\(^b\)Except for the first run (shown above), only 0.10M Glycine-HCl at pH 2.3 was used.
Figure 4.9: Immunoaffinity Purification of Bovine Plasma FXIII-Specific IgY (Two-step Method Step 2)

**Chromatography conditions:**

Temperature: Room temperature
Resin: Actigel ALD-Superflow (4.9 mg antigens/3 ml resins)
Bed dimensions: 3 ml (vol)
Packing flow rate: Gravity
Starting material: Crude IgY (28 mg at 2.7 mg/ml)
Starting buffer: 0.14M NaCl in 0.01M PB (PBS) at pH 7
Eluting buffer: 0.05M Glycine-HCl at pH 2.8, 0.10M Glycine-HCl at pH 2.3
Neutralizing buffer: 0.5M PB at pH 8
(1 part neutralizing buffer per 9 parts column effluent for pH 2.8 elution and 14 part neutralizing buffer per 86 parts column effluent for pH 2.3 elution)
Fraction size: 2 ml

**Chromatograms:**

![Chromatogram](image)

aEnzyme rich fractions (PGUB) obtained from Protein G affinity chromatography (see Figure 4.6)
Table 4.10: Immobilization of Specific IgY onto Actigel ALD-Superflow\textsuperscript{a} (IAC-II)

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Absorbance at 280 nm</th>
<th>[Protein] (mg/ml)\textsuperscript{b}</th>
<th>Total Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>15</td>
<td>0.25</td>
<td>0.18</td>
<td>2.66</td>
</tr>
<tr>
<td>Wash schedule after immobilization:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effluent during packing</td>
<td>16.5</td>
<td>0.04</td>
<td>0.03</td>
<td>0.46</td>
</tr>
<tr>
<td>0.1M PB at pH 7</td>
<td>5.0</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>0.5M NaCl PBS at pH 7</td>
<td>5.0</td>
<td>0.001</td>
<td>0.001</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.001</td>
<td>0.001</td>
<td>0.00</td>
</tr>
<tr>
<td>0.05M glycine-HCl at pH 2.8</td>
<td>5.0</td>
<td>0.01</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Mass balance (mg):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starting material</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Unbound</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bound</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Binding</td>
<td>79.9%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Bed volume = 2 ml
\textsuperscript{b}Extinction coefficient = 14 cm\textsuperscript{-1} %\textsuperscript{-1}
4.5 Immunoaffinity Chromatographic Purification of Bovine Plasma FXIII

The results obtained from both immunoaffinity columns (see Section 4.4) are shown in Table 4.11.

Purification of bovine plasma FXIII using IAC-I was deemed unsatisfactory. Several reasons could have contributed to these results: First, during the first run the column might have been overloaded with antigens (10 ml HFSN at 23 mg/ml). As a result, only the very strong binding ones remained. These antigens (not eluted by 0.05M glycine-HCl at pH 2.8) prevented further binding through the blockage of specific IgY sites either directly or via steric hindrance. In fact, it was only after two washes with 0.1M glycine-HCl at pH 2.3 that bovine plasma FXIII was successfully captured by the column and was eluted. After that, only pH 2.3 eluent was used. The average specific activity; purification factor and yield obtained during this step were 28.5 ± 0.95 units/mg protein, 7.08 ± 0.75 -fold and 6.37 ± 1.0% respectively (n=3). These values were comparable or lower than those obtained via a combination of ion-exchange and Protein G affinity chromatography (see Section 4.3).

To investigate the effect of 0.1M glycine-HCl (pH 2.3) eluent on enzyme activity, elution conditions were simulated by passing a small amount (1 ml) of HFSN through a PD-10 Sephadex G-25 column previously equilibrated with the eluent. More eluent was then added to displace the material from the column, which was immediately neutralized in the same manner as in immunoaffinity chromatography. This procedure, in contact time comparable to that observed during immunoaffinity chromatography, destroyed essentially all bovine plasma FXIII activity. Also as mentioned earlier, reducing SDS-PAGE (with silver-staining) revealed the presence of possibly several proteins including
BSA in the eluted fractions obtained from IAC-I (Figure 4.10 and APPENDIX II). These contaminants, along with possible inactivation of bovine plasma FXIII under the harsh condition of elution, as well as column overloading might have accounted for the relatively low specific activity, purification factors and yields observed.
Figure 4.10: Reducing SDS-PAGE (with Silver-staining) of Eluted Fractions from IAC-I During the Immunoaffinity Chromatographic Purification of Bovine Plasma FXIII

(a) = Eluted fractions from IAC-I

(b) = SigmaMarkers (High-Range)
Table 4.11: Results from Immunoaffinity Chromatographic Purification of Bovine Plasma FXIII

<table>
<thead>
<tr>
<th>Immunoaffinity column</th>
<th>Run no.</th>
<th>Amount of starting material loaded (mg)</th>
<th>Specific activity of starting material (unit/mg/30 min)</th>
<th>Total protein in pooled eluted fractions (mg)</th>
<th>Specific activity of pooled eluted fractions (unit/mg/30 min)</th>
<th>Purification factor (n-fold)</th>
<th>Yield (%)^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAC-I</td>
<td>1</td>
<td>85.4</td>
<td>4.40</td>
<td>0.804^a</td>
<td>29.4^a</td>
<td>6.68</td>
<td>6.28</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>102</td>
<td>3.58</td>
<td>0.964^a</td>
<td>28.5^a</td>
<td>7.95</td>
<td>7.48</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>109</td>
<td>4.15</td>
<td>0.880^a</td>
<td>27.5^a</td>
<td>6.61</td>
<td>5.36</td>
</tr>
<tr>
<td>IAC-II</td>
<td>1</td>
<td>188</td>
<td>3.97</td>
<td>0.276^b</td>
<td>199^b</td>
<td>50.2</td>
<td>7.36</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>188</td>
<td>4.87</td>
<td>0.188^b</td>
<td>316^b</td>
<td>64.8</td>
<td>6.49</td>
</tr>
</tbody>
</table>

^aPooled fractions eluted with 0.10M Glycine-HCl (pH 2.3).

^bPooled fractions eluted with 0.05M Glycine-HCl (pH 2.8).

^c[Protein] for eluted pool was determined using Bio-Rad (Standard) method with BSA whereas for starting material, Biuret microassay was employed and also with BSA as standard.

^dYield = % total activity (specific activity × total protein) of eluted pool relative to total activity of starting material loaded.
The average purification factor with respect to bovine plasma FXIII using IAC-II was 57.5 (Table 4.11). This suggests the importance of the removal of BSA cross-reactive IgY prior to column preparation. Figure 4.11 shows the typical conditions employed and the chromatogram obtained. The pooled eluted fractions yielded several bands upon reducing SDS-PAGE (with silver-staining) (Figure 4.12). PhastImage gel analyzer revealed the presence of species with apparent molecular weights of ~170, 150, 85, 69, 56 and 47 kDa and four other minor bands (APPENDIX II). The bands(s) that are most likely to be representative of bovine plasma FXIII would be those corresponding to an apparent molecular weight of 85 and/or 69 kDa.
Figure 4.11: Immunoaffinity Purification of Bovine Plasma FXIII (IAC-II)

**Chromatography conditions:**

- **Temperature:** Room temperature
- **Resin:** Actigel ALD-Superflow (2.1 mg bound IgY / 2 ml resins)
- **Bed dimensions:** 2 ml
- **Operating flow rate:** 1 ml/min
- **Starting material:** HFSN (12 ml at 15.636 mg/ml)
- **Starting buffer:** 0.05M Tris-HCl at pH 7
- **Eluting buffer:** 0.05M Glycine-HCl at pH 2.8, 0.10M Glycine-HCl at pH 2.3
- **Neutralizing buffer:** 0.5M Tris-HCl at pH 9 (0.2 ml neutralizing buffer per 1.8 ml column effluent for pH 2.8 elution; 0.24 ml neutralizing buffer per 1.76 ml column effluent for pH 2.3 elution)
- **Fraction size:** 2 ml

**Chromatogram:**

![Chromatogram](image-url)
Figure 4.12: Reducing SDS-PAGE (with Silver-staining) of Starting Material and Eluted Fractions from Immunoaffinity Chromatographic Purification of Bovine Plasma FXIII Using IAC-II

Lane 1 = SigmaMarkers (High-Range)
Lane 2-4 = Eluted fractions from IAC-II
Lane 5-6 = Starting material for IAC-II
4.6 General Discussions

It may be hypothesized that the purity of PGUB with respect to bovine plasma FXIII being used to purify the enzyme-specific IgY determines the specificity of the resulting immunoaffinity column. The fact that comparable purity was achieved by IAC-I and the ion exchange/Protein G affinity chromatography combined method was not surprising (specific activity obtained was 28.5 ± 0.95 (n=3) with the former and 34.2 units/ mg/ 30 min with the latter). The performance of an immunoaffinity column is dependent on the specificity of the IgY present, which is in turn a direct result of the purity of the ligand, i.e., PGUB (from which specific antibodies were obtained).

It was observed that BSA fractionated alongside bovine plasma FXIII into the eluted fractions during the immunoaffinity chromatographic purification of the enzyme using IAC-I. This necessitated an additional step be introduced to further improve the purity of specific IgY preparations. Results obtained herein demonstrated that the crude IgY preparations used in this work contained BSA cross-reactive IgY and that subtractive immunoaffinity chromatography using immobilized BSA facilitated their removal (Table 4.9). The specific IgY preparation (SIGY2) obtained from the two step method was much more specific towards bovine plasma FXIII, judging by the specific activity and purification factor achieved with IAC-II (Table 4.11).

There are several possible sources of BSA cross-reactivity in the IgY preparations. First, BSA might have been a contaminant present in the original material (LEE) used for immunization. The hens subsequently developed antibodies against both bovine plasma FXIII and BSA. Secondly, the procedures employed herein for IgY extraction and subsequent purification using immobilized bovine plasma FXIII (FC) was
not adequate for isolating specific IgY primarily against bovine plasma FXIII. The latter, as mentioned earlier may be due to impurities in PGUB used to construct the FC. Thirdly, the specific IgY preparations were polyclonal by nature and therefore contain multiple immunoglobulins responding to different epitopes (antibody binding sites) on bovine plasma FXIII. Part(s) of the macromolecular structure of BSA may be of close enough resemblance to these epitopes that it too may bind to bovine plasma FXIII-specific IgY.

Regarding the recovery of enzyme activity, the use of IAC-I and IAC-II resulted in yields (specific over total crude IgY) of 0.883 and 0.232 mg protein eluted respectively. Since the capacity of the columns were not known (this may be estimated by loading pure bovine plasma FXIII onto the columns and determining the amount of bound enzymes), the relatively low yields associated with immunoaffinity chromatography might have been due to mere overloading of starting material. If one assumes the molecular weights of bovine plasma FXIII and IgY are 320 (McDonagh, 1994, for human plasma FXIII) and 180 kDa respectively and that IgY has a valence of 2 (Akita and Li-Chan, 1997), the theoretical amounts of enzyme obtainable by IAC-I (4.2 mg immobilized specific IgY/ 2 ml resin) and IAC-II (2.1 mg immobilized specific IgY/ 2 ml resin) would be 14.9 and 7.47 mg, which are much higher than those obtained experimentally.

Having established that IAC-II produced the best purification with regard to specific activity and purity of the enzyme achieved in this work, it is appropriate to compare these results to what has been reported in the literature. Several methods for the purification of plasma FXIII (mostly of human origin) have been published or even patented. Other types of transglutaminase have also been isolated using a variety of
techniques. Some examples of these are shown in Tables 4.12 and 4.13. Lastly, human plasma FXIII and its catalytic units, microbial transglutaminase, as well as guinea pig liver transglutaminase are available from commercial sources. In particular, guinea pig liver transglutaminase is supplied by Sigma Chemical Co. at a specific activity of 1.5-3 units per mg protein (one unit being defined as the amount of enzyme activity that catalyzes the formation of 1 μmole of hydroxamate per min from N-α-CBZ-Gln-Gly and hydroxylamine).

It is rather difficult to directly compare purification results with respect to transglutaminase, such as specific activity or purification factor achieved among those listed in Tables 4.12 and 4.13 due to differences in the activity assay methods employed. For example, the specific activity values for erythrocyte transglutaminase purified by Ando et al. (1987) and Signorini et al. (1988) differed markedly from each other, possibly due to the different activity assay methods employed (Table 4.13). Yet, the purification factors reported were relatively similar. In both cases, the enzyme was purified to homogeneity as defined by showing a single band upon SDS-PAGE.

In addition, Ikura et al. (1987) reported that bovine plasma FXIII (catalytic units) purified from citrated plasma and from citrated plasma that had undergone ammonium sulphate precipitation and heating (to facilitate the removal of fibrinogen), when analyzed by the same assay method, yielded different specific activity results (Table 4.12).

Notwithstanding these difficulties, the following trends were observed. First, the specific activity of HFSN was comparable to that of citrated bovine plasma reported by Ikura et al. (1987). However, it was lower than that of human plasma (Cooke and Holbrook, 1974, McDonagh et al., 1976). This may indicate a lower transglutaminase
Table 4.12: Partitioning Results of Plasma FXIII Reported in the Literature
<table>
<thead>
<tr>
<th>Source</th>
<th>Transglutaminase (U/mL)</th>
<th>Specific Activity (units as Factor)</th>
<th>Identity (Identity)</th>
<th>出版 enzyme (units as factor)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kumazawa et al. (1996)</td>
<td>1.0 mg/mL</td>
<td>1.0 mmol MD</td>
<td>per min</td>
<td>transglutaminase extract</td>
<td>Human Platelet</td>
</tr>
<tr>
<td>Kumazawa et al. (1997)</td>
<td>1.0 mg/mL</td>
<td>1.0 mmol MD</td>
<td>per min</td>
<td>transglutaminase extract</td>
<td>Japanese oyster</td>
</tr>
<tr>
<td>Kumazawa et al. (1997)</td>
<td>1.0 mg/mL</td>
<td>1.0 mmol MD</td>
<td>per min</td>
<td>transglutaminase extract</td>
<td>Japanese oyster</td>
</tr>
<tr>
<td>Audo et al. (1987)</td>
<td>1.0 mg/mL</td>
<td>1.0 mmol MD</td>
<td>per min</td>
<td>transglutaminase extract</td>
<td>Human Platelet</td>
</tr>
<tr>
<td>Sugiorni et al. (1988)</td>
<td>1.0 mg/mL</td>
<td>1.0 mmol MD</td>
<td>per min</td>
<td>transglutaminase extract</td>
<td>Human Platelet</td>
</tr>
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Table 4.13: Purification Results of Transglutaminase from Various Sources Reported in the Literature.
(bovine plasma FXIII) content relative to other proteins present in Fibrimex than human plasma FXIII content in human plasma. In addition, the purification factor obtained using IAC-II (57-fold) was comparable to that obtained by Ikura et al. (1987) with crude bovine plasma FXIII preparation (44.2-fold) as well as that reported by Ando et al. (1987) for human platelet FXIII (44.1-fold). However, both Ikura et al. (1987) and Ando et al. (1987) showed that their purified enzyme was homogenous upon electrophoresis. Homogeneity was not achieved in the present study (Figure 4.12). This may be due to the lower specific activity in the starting material, Fibrimex. Therefore, the same degree of purification did not, in the present study, result in the same degree of purity as in Ikura et al. (1987) and Ando et al. (1987). Nonetheless, one must consider the limitations of assessing product homogeneity by means of electrophoresis. For instance, the presence of contaminant(s) with molecular weight may be so close to that of target protein that the two are not readily resolved. Ikura et al. (1987) used monoclonal antibody against the noncatalytic b subunits of bovine plasma FXIII to selectively isolate the catalytic a subunits. Human platelet FXIII was fractionated by Ando et al. (1987) from crude extract of plasma using a combination of ion-exchange, size exclusion and hydrophobic chromatography. Relative to these methods, the technique described herein, that is, immunoaffinity chromatography using polyclonal IgY was simpler and less time-consuming to develop and perform.

Thirdly, the use IAC-II for the purification of bovine plasma FXIII resulted in lower yields than for other methods reported in the literature. This may be attributed to (i) overloading of starting material, (ii) the relatively low specific activity of the purified
enzyme, and (iii) the eluting conditions employed (0.05M glycine-HCl, pH 2.8) during immunoaffinity chromatography, which may be denaturing to the enzyme.
CHAPTER V

CONCLUSIONS

A novel method for purifying bovine plasma FXIII has been developed and described herein. It involves the use of bovine plasma FXIII-specific egg yolk immunoglobulin (IgY) in immunoaffinity chromatography. Crude IgY preparations, of the polyclonal kind, required further purification in order to be used to construct the immunosorbent needed. In the present study, a simple method combining anion-exchange and Protein G affinity chromatography was first developed and used for purifying bovine plasma FXIII for the purpose of isolating its specific antibodies. In this regard, Protein G affinity chromatography was shown to be a useful tool for the fractionation of immunoglobulins as contaminants. It was demonstrated that bovine plasma FXIII thus purified can be used for isolating its specific IgY. However, purity of the specific IgY subsequently prepared may be enhanced by first removing IgY suspected to be cross-reactive with BSA. The immunosorbent containing bovine plasma FXIII-specific IgY purified in the two-step method produced the best purification of the enzyme with respect to specific activity (199-316 nmol MDC incorporated/ 30 min/ mg protein) and purification factor (~57 -fold). Relatively low yields were obtained in the present study. This might be due to the potentially denaturing eluting conditions employed, antigen-antibody interactions that were resistant to the eluents used, and column overloading.

The following may be considered if one should seek to further improve on this new method for the purification of bovine plasma FXIII described herein:
(i) The purity of the antigens used to obtain bovine plasma FXIII-specific IgY may be increased by additional purification, such as gel filtration, following Protein G affinity chromatography.

(ii) The effects of 0.05M glycine-HCl (pH 2.8) on the enzymatic activity of bovine plasma FXIII may be investigated. Should it turn out that this eluent reduces or destroys the activity significantly, the use of a milder eluent should be considered. Preliminary experiments indicate ActiSep, a commercial 'non-denaturing' eluent, do not inactivate bovine plasma FXIII to the same degree as 0.10M glycine-HCl (pH 2.3) (results not shown). ActiSep has been used in other immunoaffinity chromatography protocols for the purification of anti-lactoferrin IgY, lactoferrin, and anti-bovine IgG IgY (Li-Chan et al., 1998; Akita and Li-Chan, 1997). Ikura et al. (1987) used high calcium ion buffer to dissociate the a subunit from the b subunit, which contains the epitope for monoclonal antibody binding. This elution technique may be incorporated into the method developed herein, provided that immobilized specific IgY also binds to bovine plasma FXIII through the b subunit.

Immunoaffinity chromatographic method using specific IgY of the polyclonal kind for purification of bovine plasma FXIII should remain a viable and attractive option with respect to performance and ease of use, albeit further optimization is necessary.


Pierce Chemical Co. 1988. *Instructions for ImmunoPure (G) Immobilized Protein G.* Pierce Chemical Co., Rockford, IL, USA.


APPENDIX I

LogMW vs Rf Plots for Figure 4.2B

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MW Markers: SigmaMarkers (High-Range)

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![Graph showing LogMW vs Rf plots](image)
LogMW vs Rf Plots for Figure 4.3B

Lane: 1
MW Markers: SigmaMarkers (Wide-Range)

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![LogMW vs Rf Plot](image)
LogMW vs Rf Plots for Figure 4.4B

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MW Markers: SigmaMarkers (Wide-Range)

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LogMW vs Rf Plots for Figure 4.10

Lane: (b)
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LogMW vs Rf Plots for Figure 4.12

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LogMW vs Rf Plot for Figure 4.12

- LogMW
- Linear (LogMW)
### APPENDIX II

**Apparent MW of protein Bands for Figure 4.2B**

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**Apparent MW of protein Bands for Figure 4.3B**

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