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Isolation and Purification of Chicken Egg Yolk Immunoglobulins: A Review

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Isolation and purification methods for immunoglobulins from hen egg yolk are reviewed. These methods consist of a removal of most of the lipoproteins in order to obtain a water-soluble protein fraction, which can be regarded as an immunoglobulin concentrate. Water dilution methods and the use of particular anionic polysaccharides seem to offer the best IgY recoveries. Moreover both can be applied easily in an industrial environment. The further purification of the immunoglobulin can be achieved using precipitation, chromatographic or filtration methods. Filtration technology seems to offer the best opportunities for industrial applications while precipitation with polyethylene glycol or salts offer a cheap and easy methodology for laboratory use.

Keywords: *IgY, isolation, purification, egg yolk, immunoglobulin*

INTRODUCTION: POTENTIALS OF CHICKEN IMMUNOGLOBULINS

Egg yolk proteins are distributed in two particular parts: the granules and the plasma in which the former are suspended. Granule proteins are composed of α - and β -lipovitellines (70%), phosvitine (16%) and low-density lipoproteins (12%) (Burley & Cook, 1961). Some of these proteins are very important because of their functional characteristics (Baldwin, 1986). The plasma proteins consist of the α -, β - and γ -livetins and low density proteins (McCully *et al.*, 1962). The α - and β -livetins were identified as chicken serum albumin and α_2 -glycoprotein, respectively (Hatta *et al.*, 1990). The γ -livetins are the chicken immunoglobulins, which are secreted from the blood plasma into the ripening egg follicle (Lösch *et al.*, 1986). In fact, egg yolk immunoglobulins correspond to the blood serum IgG immunoglobulins and are known as IgY (Leslie & Clem, 1969). The other blood serum immunoglobulins, IgM and IgA, are found dominantly in egg white (Rose *et*

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al., 1974) but were found in the yolk as well in very low concentrations due to a possible protein diffusion from the white into the yolk sac (Lösch *et al.*, 1986). Quantitatively spoken only IgY is relevant in eggs since IgA and IgM concentrations in the egg white are considerably lower than the IgY concentration in the yolk (10 mg ml⁻¹ yolk vs. <0.1 mg ml⁻¹ egg white, Lösch *et al.*, 1986; Otake *et al.*, 1991).

The interest for IgY isolation arises from the possible applications of these immunoglobulins in diagnostics and therapeutics. Moreover they have immunoprophylactic potential (Schade & Hlinak, 1993). Therapeutic and prophylactic applications may be possible in animal production and in the treatment or prevention of human intestinal diseases (Lösch *et al.*, 1986; Akita & Nakai, 1992). Oral administration of specific egg yolk antibodies towards gastrointestinal infections by *Escherichia coli* (Ikemori *et al.*, 1992; O'Farrelly *et al.*, 1992), *Salmonella enteritidis* (Peralta *et al.*, 1994) and murine rotavirus (Bartz *et al.*, 1980) in animal models is already described. Similarly the passive immunization of infants by supplementing food with specific antibodies from the colostrum of immunized cows against *E. coli* (Hilpert *et al.*, 1977; Tacket *et al.*, 1988), rotavirus (Ebina *et al.*, 1985; Brussow *et al.*, 1987) or *Shigella flexneri* (Tacket *et al.*, 1992) is known. Similarly egg yolk immunoglobulins of immunized hens can be applied in the fortification of infant foods as suggested by Akita and Nakai (1992) and in special cases of foods as well (Lösch *et al.*, 1986).

Apart from their use as a kind of functional food or feed ingredient, specific polyclonal antibodies can be applied to almost all immunologically-based diagnostic methods such as enzyme- (Bar-Joseph & Malkinson, 1980; Khil'ko *et al.*, 1989) and radioimmunoassay (Viera *et al.*, 1986), immunoprecipitation (Song *et al.*, 1985), immunofluorescence (Doller *et al.*, 1987) etc. Most of the applications refer to their use in enzyme immunoassays for microbiological (Rose & Mockett, 1983; Ricke & Schaeffer, 1990) or chemical (Fertel *et al.*, 1981; Meisel, 1990) analysis. Hereby it should be stressed that chicken antibodies have some supplementary advantages to mammalian antibodies because they lack reactivity with Fc receptors, complement and rheumatoid factors and human anti-mouse IgG antibodies. Consequently well known interferences in immunoassays can be avoided (Larsson *et al.*, 1993; Kricka, 1999).

Despite of the fact that chicken egg yolk immunoglobulins are currently not used at their full potential, they possess a large number of advantages compared to their mammal analogues. The use of chickens for specific immunoglobulin production is more convenient compared to the use of mammals, because the antibodies are conveniently delivered in an egg and consequently no invasive techniques are necessary to harvest them. Therefore, no bleeding of the animal is necessary which is beneficial for animal welfare (Polson *et al.*, 1980; Hassl & Aspöck, 1988; Svendsen *et al.*, 1995). Poultry have moreover a lower phylogenetic status than mammals and it is therefore desirable to use birds instead of mammals (Svendsen *et al.*, 1995). Consequently a better compatibility with modern animal protection regulations is assured (Akita & Nakai, 1992). Within this respect it is worthwhile noticing that the ECVAM report recommends the use of chicken antibodies to mammalian antibodies for ethical purposes (Schade *et al.*, 1996). Antibody production is more economical (Polson *et al.*, 1980; Hassl *et al.*, 1987; Svendsen *et al.*, 1995) because of the higher immunoglobulin production, cheaper housing, the chickens lower susceptibility for diseases and because the production could proceed in commercial egg production units. A supplementary advantage is the evolutionary distance from mammals, which offer the possibility to produce specific antibodies towards for example mammalian antigens (Jensenius *et al.*, 1981).

The reason for their restricted use is the problem of IgY isolation and purification from the complex egg yolk matrix. Knowledge of these methods is indispensable for the successful implementation of immunoglobulins in one of the above mentioned possible applications. Moreover from this knowledge, existing methods can be optimised or new methods can be developed, which would encourage IgY application at a larger scale. Therefore in this paper an overview of the most important isolation and purification methods is presented.

ISOLATION AND PURIFICATION METHODS

The methods described generally consist of two parts. Firstly a water soluble fraction (WSF) is isolated containing the immunoglobulins apart from contaminating proteins. This can be considered as an immunoglobulin concentrate. A second step consists of the further purification of this concentrate or even the isolation of specific immunoglobulins to obtain an immunoglobulin isolate.

Flow sheets of the most important methods are shown in Figure 1. As can be seen, methods start with isolated egg yolk. In most of the papers, manually separated egg yolk was used, which ensures a complete removal of the egg white. Only in a couple of papers (Fichtali *et al.*, 1992, 1993) industrially separated egg yolk has been used. This yolk can be contaminated up to about 20% with egg white, which makes the isolation and purification process somewhat more difficult (Fichtali *et al.*, 1992).

CRUDE EXTRACTION OF IMMUNOGLOBULINS FROM EGG YOLK

From the flow sheets given in Figure 1, it can be concluded that basically five general methods are available which are all based on the precipitation of the yolk granules. These methods are summarized in Table 1. By this precipitation, the IgY, together with some other proteins is left in the WSF.

Jensenius *et al.* (1981) originally introduced the water dilution of the egg yolk as a simplification of their dextran sulphate method. The yolk lipids aggregated at low ionic strength (10-fold dilution of the egg yolk with water) at a pH of 7 or lower after freezing and thawing. After centrifugation, enough lipids were removed to make further purification steps possible. Instead of including a freeze and thaw step, Kwan *et al.* (1991) incubated the aqueous dilution overnight at pH 6 (unadjusted) and pH 7. More lipids were removed at the lower pH and by increasing the dilution ratio. On the other hand however, too high dilutions induced a loss of proteins in the WSF as well.

Akita and Nakai (1992) studied the aqueous dilution method in further detail. Low ionic strength seemed to be indispensable for aggregation of the lipids. Lowering the pH below 5.0 decreased immunoglobulin recovery from 93 to 75%, while a pH increase up to 6.6 caused even a more dramatic loss (55% recovery). At a pH level of 5–5.2, lipid recovery in WSF attained a minimal level and IgY recovery was maximal. In a further study, the Nakai group tried to implement the water dilution method for a large scale operation (Kim & Nakai, 1996). Therefore the incubation time was shortened to 2 h and a freeze-thaw cycle was included in order to enhance granule coagulation. The supplementary freeze-thaw cycle seemed to be necessary since Horikoshi *et al.* (1993) revealed that shorter incubation times induced a higher residual lipid content of the WSF.

TABLE 1. Summary of the IgY isolation methods

Water dilution method:
<ul style="list-style-type: none"> ● with/without pH adjustment ● with/without freeze/thaw cycle
Polyethyleneglycol precipitation
Anionic polysaccharide precipitation
Organic solvent extraction/precipitation
Interaction with specific chemicals
<ul style="list-style-type: none"> ● caprylic acid ● hydroxypropylmethylcellulose ● phosphotungstic acid/magnesiumchloride

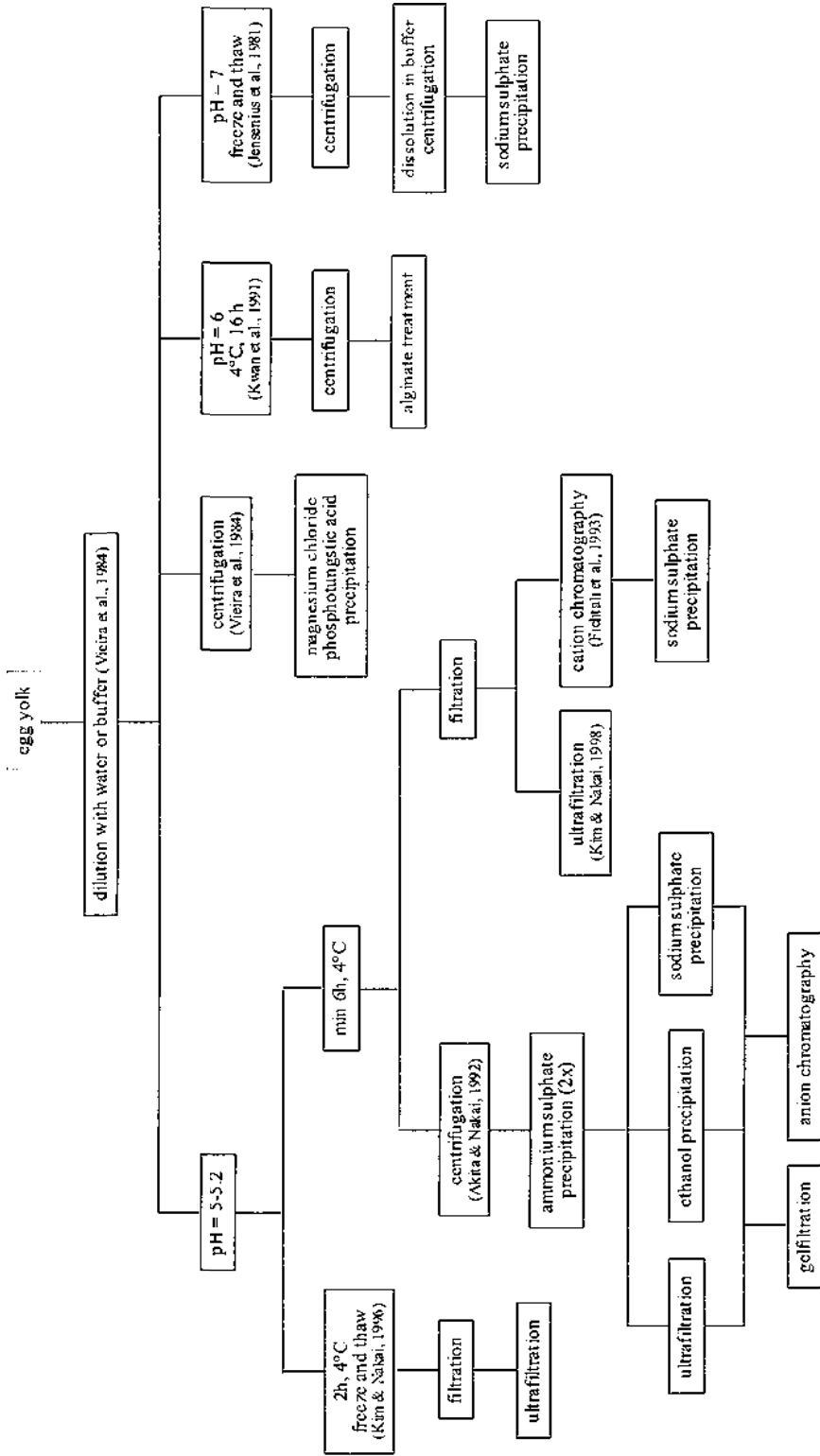


FIG. 1(a). IgY isolation and purification flowsheet using aqueous dilution in isolation step.

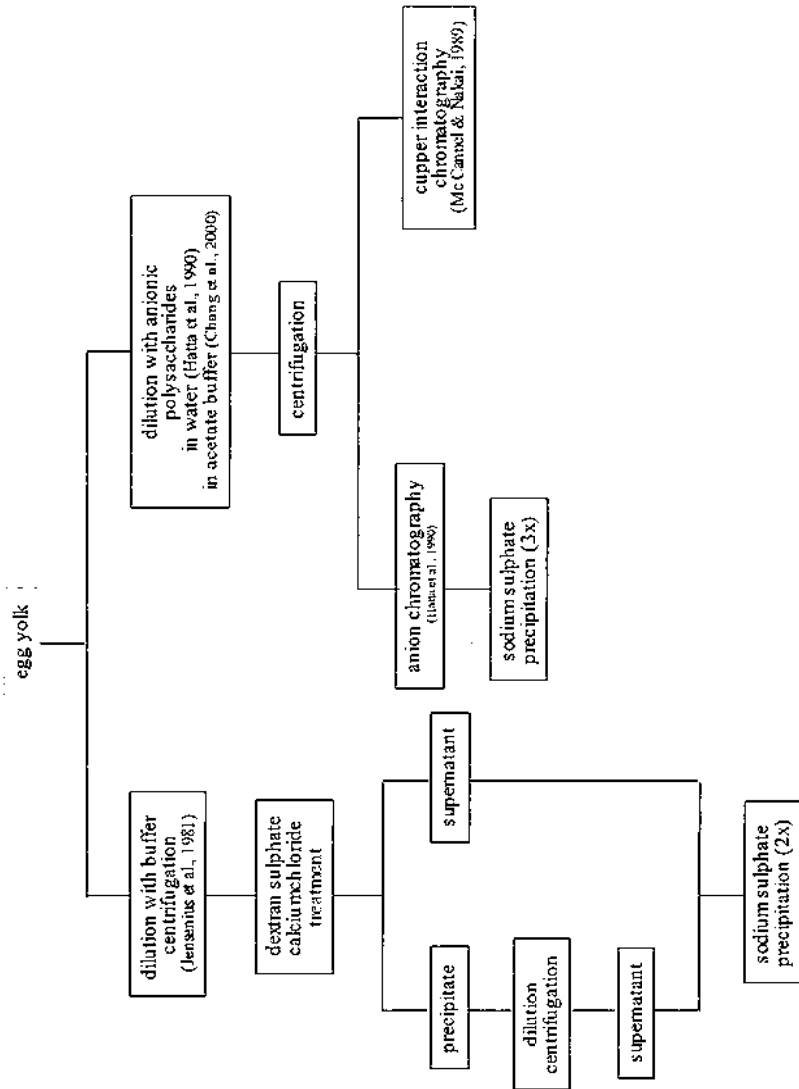


FIG. 1(b). IgY isolation and purification flowsheet using anionic polysaccharides in isolation step.

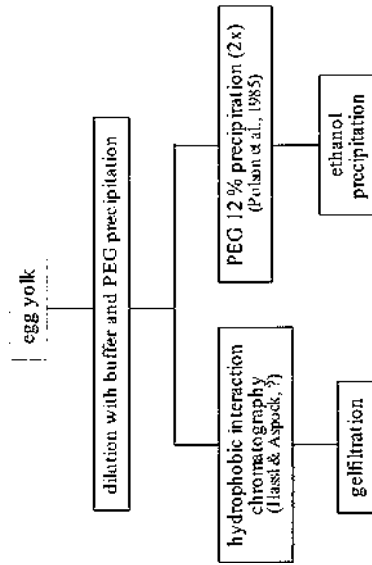


FIG. 1(c). IgY isolation and purification flowsheet using PEG precipitation in isolation step.

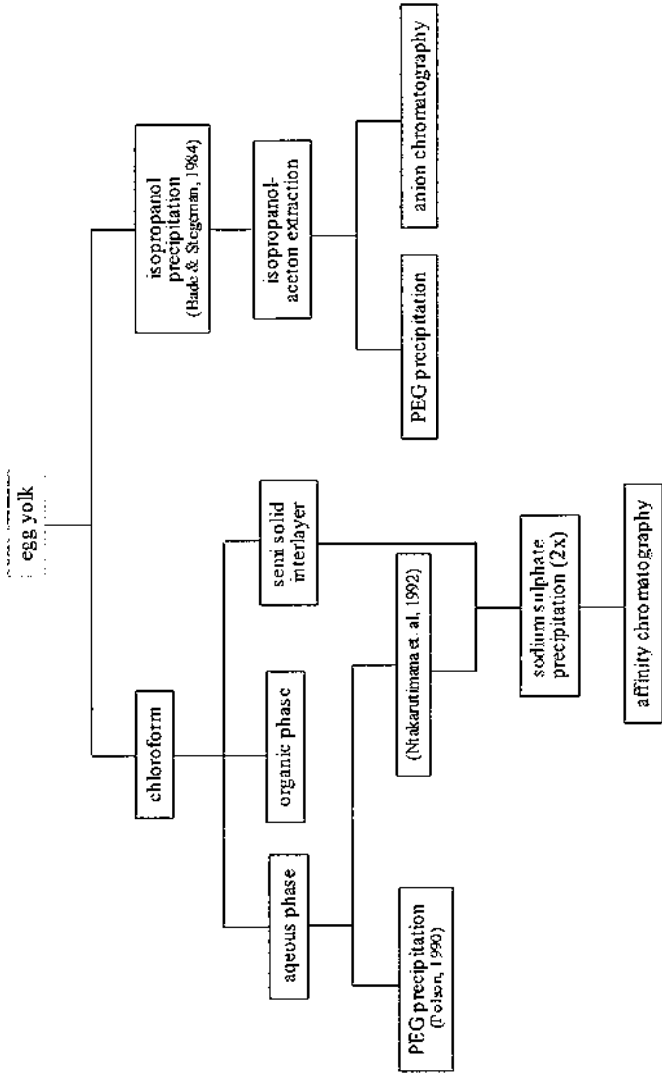


FIG. 1(d). IgY isolation and purification flowsheet – using organic solvents for isolation step.

For the sake of completeness it should be mentioned that using fresh eggs, higher lipid contamination of the WSF is observed if no lowering of the pH is applied. Most probably this is due to the stronger non covalent lipoprotein-lipid interaction (Akita & Nakai, 1992). Older eggs on the other hand are more difficult to handle since the yolk membrane becomes more fragile.

Polson *et al.* (1980) were the first to introduce the use of polyethylene glycol (PEG) in the extraction of IgY from egg yolk. They improved their method (Polson *et al.*, 1985) by increasing the dilution with buffer from a 2:1 to a 4:1 ratio. Polson (1990) rejected later on this method because it is time consuming and moreover yields are very low. This was because the precipitate obtained after the first PEG treatment (Figure 1(c)) contained over 50% of the total IgY content of the total yolk. Moreover, IgY aggregates are formed (Hassl *et al.*, 1997). Jensenius *et al.* (1981) applied the interaction between the anionic polysaccharide dextran sulphate with egg yolk lipoproteins to obtain a IgY concentrate. Because dextran sulphate is rather expensive, minimal amounts are generally added, sometimes necessitating a supplementary addition if no complete removal of lipoproteins is achieved (Jensenius & Koch, 1993). Calcium chloride is added in order to precipitate the excess of dextran sulphate. Recently, Bizhanov and Vyshniauskis (2000) replaced the dextran sulphate for dextran blue, without getting better results.

Hatta *et al.* (1990) systematically compared the use of various natural gums. Xanthan and carrageenan seemed to be able to remove most of the lipid, while the protein recovery was still considered as acceptable. Based on this study, McCannel and Nakai (1989, 1990) used alginates to prepare an IgY concentrate. Similarly, Kwan *et al.* (1991) suggested to use alginates to further purify the WSF obtained after aqueous dilution of egg yolk.

Chang *et al.* (2000) recently compared some anionic polysaccharides at various concentrations and pH levels. According to their opinion, the lipoprotein-polysaccharide interaction is governed by ionic bounds, hydrophobic interactions and hydrogen bounds, depending on the polysaccharides used. For alginates, all three interactions are almost equally important, while for pectin especially hydrogen bounds and ionic interactions are important. The combination pectin at a 0.15% level and pH 5 was considered to be optimal to obtain a crude IgY concentrate.

Bade and Stegeman (1984) used cooled isopropanol to precipitate the water soluble proteins and further removed lipidic material by consecutive isopropanol and acetone extractions. Temperature is a critical factor in this method because of the denaturing effect of the solvents on proteins. Comparable results with the Polson method (Polson *et al.*, 1980) were obtained.

Polson (1990) and Ntakirutimana *et al.* (1992) both used chloroform as an extraction solvent for the lipids. A complex three-layered mixture was obtained after centrifugation. It consisted at the bottom of an orange coloured organic phase, a semi-solid middle phase and a aqueous phase at the top, containing the immunoglobulins. The semi-solid phase can be extracted with buffer in order to increase IgY recovery (Ntakirutimana *et al.*, 1992). Surprisingly, active immunoglobulins were obtained although chloroform is known to be a protein denaturing agent (Polson, 1990). Moreover, better extraction of IgY was observed compared to the previous Polson method (Polson *et al.*, 1985).

Generally, however, these latter methods have the disadvantage of using organic solvents, sometimes in quite excessive amounts (Bade & Stegeman, 1984). Apart from environmental objections, use of these solvents in the isolation of possible food ingredients is to be avoided, especially if better alternatives are available.

Finally, some other chemicals were used to induce removal of the lipoproteins from the egg yolk. Caprylic acid, used before by Steinbuch and Audran (1969) for the purification of IgG from rabbit serum, was evaluated by McLaren *et al.* (1994) and Svendsen *et al.* (1995). Under mildly acidic conditions, caprylic acid precipitates most blood serum proteins except IgG. Svendsen *et al.* (1995) however concluded that caprylic acid was not able to precipitate egg yolk proteins and therefore considered it useless for IgY isolation. Yokoyama *et al.*

(1993) used hydroxypropylmethylcellulose phthalate but also here no additional advantages could be observed compared to the other methods. Finally, Vieira *et al.* (1986) successfully applied phosphotungstic acid and magnesium chloride to remove lipids in an aqueous diluted egg yolk.

Comparing the efficiency of these methods towards IgY recovery and lipid removal is difficult. Sometimes the IgY yield and purity are not specified or have been determined using different methods. Therefore, comparable studies are most appropriate for this purpose. Recently, Deignan *et al.* (2000) evaluated five methods to obtain an IgY concentrate. The dextran sulphate and phosphotungstic acid methods were both the better methods with regard to IgY recovery, quickly followed by the freeze-thaw procedure of Jensenius *et al.* (1981). Surprisingly the Polson *et al.* (1985) method seemed to give reasonable results well. The use of isopropanol and acetone (Bade & Stegeman, 1984) gave the lowest recoveries. Akita and Nakai (1993) compared their water dilution method with the PEG method of Polson *et al.* (1985) and the methods including dextran sulphate (Jensenius *et al.*, 1981) and xanthan (Hatta *et al.*, 1990). Highest yields were obtained by the water dilution method (90%), lowest by the PEG method (50%).

To conclude, it is obvious that various IgY isolation procedures exist with differing results towards recovery and purity. Methods making use of organic solvents should be avoided because of several reasons. The original PEG methodology (Polson *et al.*, 1980, 1985) can be replaced by other more efficient methods, which are moreover less labour intensive. If a minimum of chemicals is to be used, the water dilution techniques, which may include a pH correction or a freeze-thaw cycle, could be a better choice. The use of anionic polysaccharides may be a valuable alternative because these methods are quicker. On the other hand, the reagents are quite expensive and because they are natural products some batch to batch variation may be present. As already stated, caprylic acid offers no good alternative. Although the method of Vieira *et al.* (1984) seemed to perform very well, quite a lot of chemicals are consumed, which is in contrast with other methods with similar or better performances.

IGY PURIFICATION

Depending on its application purification of the immunoglobulin concentrate could be necessary. The concentrate generally contains other water soluble proteins together with some minor lipids or lipoproteins. Three kinds of separation techniques can be used to eliminate these contaminants: precipitation, chromatography and filtration (Table 2). One total clean-up procedure could include several of these techniques as indicated in Figure 1.

Salt precipitation is a traditional technique in protein purification. For IgY, especially, ammonium and sodium sulphate have been used. The concentration levels used may vary although Akita and Nakai (1992) observed a concentration dependent IgY yield and purity using ammonium sulphate. Salt precipitations are generally repeated once or even twice which increases IgY purity drastically. Also a shift from ammonium to sodium sulphate is reported. Deignan *et al.* (2000) compared the separate use of both salts, but could not find significant differences for efficiency and purity.

Polson *et al.* (1985) and Akita and Nakai (1992) used the cryoethanol method to obtain purified IgY. Ethanol concentration influenced IgY recovery (Polson *et al.*, 1985). Of course ethanol risks denaturing the proteins, so care needs to be taken in order to control the temperature (-20°C) and to thoroughly remove the ethanol. After salt precipitation cryoethanol treatment gave an IgY isolate of 93% purity (Akita & Nakai, 1992). Horikishi *et al.* (1993) proposed to use ethanol in a large scale IgY production, but the process proposed consumes quite high amounts of ethanol and includes too many centrifugation steps to be really practical in an industrial environment.

TABLE 2. Summary of the IgY purification methods

Precipitation reactions:
<ul style="list-style-type: none"> ● ammoniumsulphate ● sodium sulphate ● polyethyleneglycol ● ethanol
Chromatographic methods:
<ul style="list-style-type: none"> ● thiophylic interaction chromatography ● affinity chromatography ● anion exchange chromatography ● cation exchange chromatography ● hydrophobic interaction chromatography ● gelpermeation chromatography ● copper interaction chromatography
Filtration methods:
<ul style="list-style-type: none"> ● funnel filtration ● diafiltration ● columnfiltration ● ultrafiltration

The most successful precipitation method with respect to IgY recovery and purity consists of adding PEG at a 12% level (Akita & Nakai, 1993) as in the method of Polson *et al.* (1985). This observation is confirmed by the recent comparing study of Deignan *et al.* (2000). PEG has moreover the supplementary advantage it can be used at ambient temperatures without any risk for protein denaturation. The use of PEG implies dialysis or gel permeation in order to remove the polymer completely or the use of chloroform as suggested by Polson (1990).

Various chromatographic techniques can be used to further purify the crude IgY concentrate. Bade and Stegeman (1984) proposed to further purify their isopropanol/acetone defatted IgY extract with anion exchange chromatography using DEAE-cellulose as stationary phase. Previously, Raju and Mahadevan (1974) described the use of this stationary phase for the isolation of low density lipoproteins from egg yolk plasma. It seems, however, that if the sample applied contains to many contaminating proteins, purification is rather restricted (McCannel & Nakai, 1990) and preceding purification steps such as salt precipitation (Hatta *et al.*, 1990) are necessary in order to obtain electrophoretically pure IgY (Akita & Nakai, 1992). Moreover, sample loads are quite restricted compared to other chromatographic methods (McCannel & Nakai., 1990). For the sake of completeness it should be mentioned that Fichtali *et al.* (1993) evaluated another anionic stationary phase (HA-2, Gibco Gel) without success.

Cation exchange chromatography as proposed by Fichtali *et al.* (1992, 1993) could be successfully implemented in an automated liquid chromatographic system for the purification of industrially separated egg yolk (recovery 60–65%). Purities (60–69%) could be further improved by subsequent salt precipitation (Fichtali *et al.*, 1993).

Hassl and Aspöck (1988) evaluated the possibilities of hydrophobic interaction chromatography followed by gelpermeation chromatography to purify IgY from a PEG treated egg yolk. Yields, however, were considerably lower compared to the Polson *et al.* (1985) method. On the other hand, they stressed the usefulness of gelpermeation chromatography for buffer exchange instead of time-consuming dialysis steps.

Akita and Nakai (1992) used gelpermeation chromatography as well, but used a 1.5 M-sodium chloride solution as a mobile phase. Due to these high salt concentrations IgY

polymerized enabling collection of electrophoretically pure immunoglobulin from the void volume fraction using a Sephacryl S-200 gel.

Due to varying affinities of proteins for heavy metals (Porath *et al.*, 1975), copper loaded chelate interaction chromatography was evaluated for its applicability in IgY isolation from an alginate treated egg yolk (McCannel & Nakai, 1989). If small sample sizes were used, IgY did bind to the stationary phase enabling its isolation with a fair recovery (46%) and purity (65%). Overloading the column however resulted in a displacement of IgY to the unbound fraction resulting in an isolate with higher purity (74%) but lower recovery (30%).

Hansen *et al.* (1998) described the use of a mercaptoethanol modified Sepharose CL-4CB gel. This thiophylic interaction chromatography (Porath *et al.*, 1985) was applied on a aqueous diluted egg yolk including double salt precipitation. Thus a pure IgY isolate could be obtained with good yields (85%).

Although some of these chromatographic techniques are able to fractionate even IgY subclasses (McCannel & Nakai, 1990), affinity chromatography is the most appropriate to isolate specific antibodies from a IgY concentrate. The use of antigen affinity columns (Ntakaturima *et al.*, 1992; Kuronen *et al.*, 1997), immunosorbent columns (Yazawa *et al.*, 1991) and synthetic ligand affinity columns (Yokoyama *et al.*, 1993; Fassina *et al.*, 1998) has been reported. Elution of the bound specific antibodies from the column without affecting their activity can be achieved by for example strong acidic (Ntakaturima *et al.*, 1991) or alcalic (Yazawa *et al.*, 1991; Kuronen *et al.*, 1997) elution followed by immediate neutralization. For the acidic elution it should be stressed that more drastic conditions as those frequently applied for the purification of mammalian antibodies (e.g. 0.1 M-glycine, pH 3) should be applied. For the sake of completeness it should be mentioned as well that IgY is not bound to protein A or protein G, in contrast to mammalian antibodies. Consequently attempts to use of these proteins for the purification of chicken antibodies have been unsuccessful.

Filtration methods using traditional funnel filtration or otherwise column- and ultrafiltration offer another possibility for IgY purification. Especially the two latter techniques are appropriate for large scale industrial applications (Kim & Nakai, 1996, 1998). A WSF obtained via various water dilution methods was first delipidated using hydrophobic membrane filtration (Kim & Nakai, 1996) or a cellulose powder column in conjunction with an octadecyl column (Kim & Nakai, 1998). Subsequently IgY was polymerized in the presence of sodium chloride and purified via ultrafiltration. Both high recoveries (72–89%) and purity (74–99%) were obtained using various filtration membranes.

To conclude, PEG seemed to be the best chemical for IgY purification via selective precipitation in a laboratory environment. Salt precipitation on the other hand is a valuable alternative. Filtration techniques, especially column- and ultrafiltration have large potential for industrial applications. Some chromatographic methods could be upscaled as well, but major drawbacks in most cases are the fairly expensive stationary phases and limited sample loads. Therefore, filtration techniques seem to have better potential in an industrial environment. Some chromatographic techniques however seem to be valuable in a laboratory environment, especially if a fractionation of specific antibodies is intended.

CONCLUSION

Chicken egg yolk immunoglobulins are a good alternative for mammal immunoglobulins in various immunological diagnostic methods. Moreover, their use in functional foods may enable therapeutic and prophylactic applications. Purification methods include removal of the lipoproteins in order to obtain an IgY concentrate. Further purification may enable isolation of pure immunoglobulins. For both steps, both laboratory and industrial scaled methods are available. Till now, a limited amount of papers dealing with industrially separated egg yolk are published. Therefore, an evaluation of some of the above described methods using industrially separated egg yolk would be very interesting. Knowledge of current isolation and

purification methods of IgY from egg yolk will enhance the development of new methodologies and will encourage the IgY use in various fields as well.

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