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Extraction of IgY from egg yolk using a novel aqueous two-phase system and comparison with other extraction methods

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ABSTRACT

Egg yolk is an important source of antibodies. The biggest obstacle for isolation of chicken antibodies (IgY) is the removal of lipids, which are present in abundance in egg yolk. We have used a two-phase system to separate egg yolk. The use of an aqueous two-phase system with phosphate and Triton X-100 made separation of lipids and water-soluble proteins possible. Lipids are extracted into the detergent-enriched top-phase, whereas IgY is isolated in the phosphate-enriched bottom-phase. The phosphate:triton system was characterised and optimised using various experimental designs. For the optimised model, the yield of IgY was kept above 97% (11.1–14.9 mg IgY/g egg yolk recovered). The amount of lipids in the bottom-phase was kept below 25% of the total content in the egg yolk added. Hence, the model described provides a method for extracting the IgY-fraction with a high yield and relatively low lipid content.

INTRODUCTION

An aqueous two-phase system (ATPS) is formed when two water-based solutions give a biphasic solution when mixed. The first observation of ATPS was made more than a century ago [5]. The most common and well-known system is the one formed from a mixture of Dextran and polyethylene glycol (PEG) [2]. In these systems, the top-phase is enriched in PEG and the bottom-phase is enriched in Dextran. The concentration of polymers in each phase depends on the total concentrations of polymers in the whole system. Aqueous two-phase systems may also form on addition of salts to solutions of certain polymers [16]. PEG and phosphate is one example of such a system.

One of the first reasons for using an ATPS instead of an organic extraction when working with biological material is that partition in an ATPS occurs under conditions that preserve biological activity [3]. Another ATPS is that it does not produce any hazardous waste, in contrast to organic extractions. In fact, many of the components used in ATPS, such as PEG, phosphate and Dextran, are accepted for therapeutic purposes. After a system has settled, the wanted phase, say the top-phase, can be transferred to a pure bottom-phase. This procedure, known as counter-current distri-

bution (CCD) can greatly enhance the grade of purification that can be achieved with an ATPS [24]. One last reason for using an ATPS when isolating IgY is the ease of scaling up. No major changes have to be made when going to larger vessels. Aqueous two-phase systems on a scale up to 1000 kg biomass have been reported for the isolation of target proteins from cell debris and cell culture media [25].

In regular IgG isolation, bleeding of the animal is necessary to purify the antibodies from the blood serum. In case of IgY, the antibodies are accessible in the eggs. Bleeding is considered painful for the animals and the use of yolk antibodies is therefore recommended by the European Centre for the Validation of Alternative Methods (ECVAM) for animal welfare reasons [21]. Another reason for isolation of antibodies from egg yolk is the larger amount of antibodies that are possible to produce. An egg contains more than 100 mg IgY with specific antibody yields somewhere between 2 and 10% [21]. Since a hen produces roughly one egg per day, the amount of antibodies that can be produced from one hen is large. There is also an economical advantage in using chickens for production of antibodies in that the cost of keeping chickens is less than that of keeping rabbits. Another advantage is that chickens are available in inbred strains. This minimises the genetic variation in antibody response, which is often seen in rabbit antibody production [12].

Egg yolk contains approximately 50% water. The dry weight of egg yolk is made up of 2/3 of lipids, and 1/3 of proteins [22]. The proteins are generally divided into four fractions, lipovitellin, phosvitin, low-density lipoprotein and livetin. The IgY-fraction is a subclass of the livetins [8]. For a large-scale production, one of the problems is separating the water-soluble proteins from the lipids and other hydrophobic substances. Organic extractions are functional, though they generally give some loss of IgY due to precipitation of proteins by the organic solution [19]. The main problem with large-scale organic extractions is the large quantities of organic waste that such a method produces. The use of organic chemicals also presents problems concerning the work environment and potential fire hazards.

For an ATPS application with egg yolk the difference in partition coefficient between IgY (target protein) and lipids (impurity) should be maximised. This primary extraction step should be aimed at giving a satisfactory yield of IgY. If successful, an ATPS could be used to separate IgY into one phase and lipids into the other. If this is accomplished, specific IgY can be extracted from the IgY-phase by affinity chromatography, or the IgY-fraction can be extracted by selective precipitation or T-gel chromatography in a secondary purification step.

The purpose of this study was to find a new method of isolating the total IgY fraction from egg yolk. The goal was to find an aqueous two-phase system that would increase the yield as compared to previously published methods. The method should also be suitable for large-scale production.

MATERIALS AND METHODS

Materials

Eggs from specific pathogen free chickens were obtained from OVA Production (Morgongåva, Sweden) and kept at 4°C until used. Rabbit anti-chicken IgY and

affinity purified chicken IgY were obtained from Immunsystem AB (Uppsala, Sweden). All systems, solutions and dilutions were made with 18MW water (Milli-Q Water Purification System, Millipore Inc., Bedford, USA). Triton X-100 from Merck (Darmstadt, Germany), Dextran T-500 from Pharmacia (Uppsala, Sweden), PEG 600 from BASF (Ludwigshafen, Germany) and phosphate-salts, $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot H_2O$, from Merck (Darmstadt, Germany) were used to make the two-phase systems.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was performed under non-reducing conditions on a Pharmacia Phast System using an 8–25% gradient PhastGel (Pharmacia Biotech, Uppsala, Sweden). Protein bands were visualised either by silver staining or staining with Coomassie Brilliant Blue. The gel was analysed using a scanner (MultiImager, Bio-Rad, Hercules, USA).

Immunodiffusion – Qualitative test of non-specific IgY

Wells were stapled into a 1% agarose gel, 8 µl of sample and rabbit anti-chicken IgY were added to the wells and the plates were incubated overnight at room-temperature (RT) in a moist-chamber. Precipitation bands were visualised without staining.

Radial immunodiffusion – Quantification of non-specific IgY

Radial immunodiffusion (RID) was done essentially as described by Hudson et al. [14]. A 1% solution of agarose in PBS-azide (0.1M NaCl, 0.01M phosphate pH 7.2 with 0.02% NaN_3) was melted and kept above its melting point in a water bath set to 56°C. Rabbit anti-chicken IgY was added to give a 30-fold dilution of the antibody and the solution was poured into RID plates. Affinity-purified anti-human insulin IgY was used as a standard. 3 µl of standards and samples were added to the wells. The plates were incubated overnight at RT in a moist-chamber. The gels were soaked in PBS-azide for 2 h to remove non-precipitated proteins. The precipitation rings were then visualised by staining with Coomassie Brilliant Blue. The diameters of the rings were measured and a standard curve of area vs. concentration was made. To correct for variations in the thickness of the gels, the diameter of precipitation rings for samples were normalised against a standard included in each plate. The concentration of each sample was then derived from the standard curve.

The compatibility of the test with high concentrations of Triton, NaCl etc., was tested by dialysing the samples. The results from the RID of samples that were dialysed were compared with the results for the same samples without dialysis. The compatibility of the test was also tested by comparing the total amount of IgY in a system (sum of content in all phases) with the amount of IgY in diluted egg yolk.

Dialysis

The membrane used was a Spectra/Por 4, with a molecular weight cut-off of 12–14000 Da (Spectrum, Gardena, CA, USA). The dialysis was done against PBS-azide with dialysis volume approximately 100 times larger than the sample volume. The PBS-azide was changed twice a day for three days.

Total protein determinations

Total protein concentration was determined by measuring absorbance at 280 nm (Hitachi U-1100 Spectrophotometer, Tokyo, Japan). An extinction coefficient at 280 nm of 13.5 for a 1% solution of IgY was used to calculate the concentration [23]. Total protein concentration was also determined by a dye reagent (Bio-Rad Protein Assay, Bio-Rad, Hercules, USA) procedure according to Bradford [7], using BSA (Protein Standard II, Bio-Rad, Hercules, USA) or human IgG (Pharmacia-Upjohn, Stockholm, Sweden) as a standard. The concentration of unknown samples was derived from a standard curve. Measuring of absorbance at 280 nm is disturbed by any concentration of Triton, whereas the Bradford test is compatible with Triton X-100 concentrations up to 0.1%.

Triglyceride test – Lipid determination

A triglyceride standard (Autonorm, Nycomed, Oslo, Norway) and samples were diluted in a microtitre plate. An equal volume of triglyceride reagent (Boehringer Mannheim, Mannheim, Germany) was added to all wells. After incubation in RT for 10 min, the plate was read at 490 nm in a microplate-reader (SpectraMax 250). Known concentration of standard is plotted against absorbance. Triglyceride content of samples is derived from the generated standard curve.

Extraction methods for separating IgY from egg yolk

All systems were made up on a w/w basis. To ensure that all material was dissolved in the systems, the ATPS component which was harder to dissolve was added first. The egg yolk was added before the second component of the ATPS because it was found to be easier to get a homogenous egg solution. Other components were added in varying order to see whether the order of addition had a significant effect. Unless otherwise stated, the system was centrifuged for 10 min in RT at 1000g (Hettich Rotina 48R, Tuttlingen, Germany).

1. Polson method [20]

In this method the egg yolk was diluted 5 times in 0.1M phosphate buffer, pH 7.6. PEG was added to a concentration of 3.5% and the solution centrifuged at 5 000 g for 20 min. For calculations of yield the precipitate, which contained most of the lipids and lipoproteins, was diluted 10 times and Triton plus NaCl were added to a final concentration of 2% to get a homogenous solution. Additional PEG was added to the supernatant, to increase the concentration to 12%. The solution was centrifuged at 5 000 g for 25 min. The precipitate was diluted 50 times in phosphate buffer. Amount of IgY in each fraction and the yield in the second precipitate (F_2) was calculated from the RID results.

N.B. In the method by Polson et al. [20], the second precipitate is used for further purification. This involves one additional precipitation with PEG and finally an alcohol precipitation.

2. Akita method [1]

The method involves a 10-fold dilution of egg yolk in pre-acidified water to give an egg yolk solution of pH 5.2. The solution was mixed, incubated for 6 h at 4°C and

then centrifuged (10,000g for 1 h at 4°C). The precipitate was diluted 10 times, Triton and NaCl were added to a final concentration of 2% to get a homogenous solution. Samples of the diluted precipitate and supernatant were collected and the amounts of IgY were calculated using the RID results. This made calculation of the yield in the supernatant possible.

N.B. In the method by Akita et al. [1], the supernatant is used for further purification. Active IgY is recovered by a purification scheme, which involves salt precipitation, ultrafiltration and gel filtration.

3. PEG:Dextran system

Concentrations of Dextran and PEG ranging from 3-7% and 5–9%, respectively, were tried. The effect of adding additional components was tested for a 5% PEG, 5% Dextran system. Triton, NaCl and phosphate were added in the range 1–5%. The system was tested with egg yolk amounts of 5, 10, 15 and 20%. Phases were tested for IgY qualitatively by SDS-PAGE and immunodiffusion and quantitatively by RID.

4. PEG:phosphate system

Concentrations of PEG and phosphate ranging from 3–20% and 5–20%, respectively, were tried. The effect of adding additional components was tested for a 5% PEG, 20% phosphate system. Triton and NaCl were added in the range 1–10%. The system was tested for an egg yolk concentration of 7.5%, unless otherwise stated. Phases were tested for IgY qualitatively by SDS-PAGE and immunodiffusion and quantitatively by RID.

5. Phosphate:Triton system

Concentrations of phosphate and Triton ranging from 5–15% and 1–15%, respectively, were tried. Other detergents tested were Tween-20 and Triton X-114. The effect of adding additional components was tested on various Phosphate:Triton systems. NaCl was added in the range 1–10%, and PEG in the range 1–2%. The pH dependence of the system was also controlled by varying the mass-ratio of the buffer-salts added ($HPO_4^{2-}/H_2PO_4^-$). The pH values referred to in the text are for the phosphate and water solution, not for the total system. Various egg yolk concentrations were tested, ranging from 5 to 25%. Phases were tested for IgY qualitatively by SDS-PAGE and immunodiffusion and quantitatively by RID.

Optimisation and evaluation of extraction systems

The phosphate:Triton system was at various stages evaluated and optimised using Statistica. The response surface model (RSM) included variations in the concentration of phosphate, Triton and NaCl. The $HPO_4^{2-}/ H_2PO_4^-$ mass-ratio was set to 1.08 and the amount of egg yolk was kept constant at 16.7%. The –1 and +1 levels for the variables were set as follows; phosphate at 7.7% and 8.3%; Triton at 9.4% and 10.4 % and NaCl at 7.3% and 8.3%. A second order, central composite design was used for the optimisation (full factorial design with three factors, star points at $\pm 2^{3/4}$). Samples were taken from the total system and from the top-phase after phase-separation.

To find an operating range for the factors, a robustness test was done. All factors that were weighed in the system were set to ± 0.1% to find how sensitive the operating range was for variations in weighing. The weight of HPO_4^{2-} was set to 4.1% and 4.3%, $H_2PO_4^-$ to 3.7% and 3.9%, Triton to 9.8% and 10.0%, NaCl to 7.4% and 7.6% and egg yolk to 16.6% and 16.8%. The phosphate-concentration and HPO_4^{2-}/ $H_2PO_4^-$ mass-ratio were varied as a consequence of the variations in HPO_4^{2-} and $H_2PO_4^-$. The resulting variations in phosphate-concentration was between 7.8% and 8.2% and the HPO_4^{2-}/ $H_2PO_4^-$ mass-ratio between 1.05 and 1.16. The robustness of the method was validated by a reduced screening design plus two centre points to evaluate the lack-of-fit for the model (half-factorial design with 5 factors and two centre points).

RESULTS

1. Polson method

The first centrifugation gave a yellow precipitate that weighed about 20% of the total solution. The supernatant was clear with a slight yellowish color. The second centrifugation gave a small white pellet, which gave a slightly cloudy solution when diluted 50 times in phosphate buffer (0.1M phosphate, pH 7.8). For the desired phase (F_2), the amount of IgY was between 9.1 and 10.4 mg IgY/g egg yolk. Relating back to the average value for the "total" system for each batch, this gave a yield of 71.2% for the method. The experiment was done 5 times with a relative standard deviation of 4.7% for the yield. The yield and the fact that the final volume of F_2 was rather small made the method interesting. The concentration of IgY in F_2 could probably be increased by decreasing the dilution factor (dilution factor of 50 used in experiments).

The F_2 phase contained 0.14% of the total triglycerides. The results for the total amount of triglycerides in the system corresponded well with that of diluted egg yolk. The method thus gave a very good separation of IgY and lipids (more than 99.8% of the triglycerides were removed). The total protein concentration of F_2 was 3.50 mg/ml as determined by a Bradford test. Relating back to the concentration of IgY given from RID, the purity of IgY was 92%. The high purity of IgY implied that the method could be used for isolation of the IgY-fraction without affinity purification.

In conclusion, the method gave a satisfactory yield of IgY, which was well separated from lipids and other proteins. The two centrifugation steps, the higher cost and the fact that the method was work intensive made the Polson method less suitable for use on a large scale.

2. Akita method

Centrifugation of the egg yolk solution gave a yellow precipitate that weighed about 13% of the total solution (w/w). The supernatant was clear with a slight yellow color. Concentrations and amounts of IgY in each phase were derived from RID as stated earlier. The wanted phase (SN) gave amounts of IgY ranging from 9.5 to 10.8 mg IgY/g egg yolk added. The method gave an average yield of 71.8% in the wanted phase. The method was repeated 5 times with a relative standard deviation of 7.8%

of the yield. The amount of triglycerides was 0.325% in the wanted phase. This indicated that the method gave a good purification of water-soluble proteins from lipids. The total protein concentration was 2.92 mg/ml, as determined by a Bradford test. Relating back to the concentration of IgY given from RID, the purity of IgY was 38%. This low purity was expected since the method did not involve any step to discriminate between water-soluble proteins and IgY. To increase the purity, the method would have to be combined with either a PEG-precipitation for isolation of the IgY-fraction or affinity purification for isolation of specific IgY.

To sum up the Akita method, it gave a satisfactory yield of IgY, with good separation from lipids. The disadvantages were that the method required centrifugation steps and that it gave large volumes for affinity purification since the dilution of egg yolk was higher.

3. PEG: Dextran system

Independent of concentration of additional components (such as Triton, NaCl and phosphate) added to the system, the selectivity of the system was not satisfactory. The lipids and IgY always partitioned in the same phase.

4. PEG: phosphate system

Independent of concentration of additional components (such as Triton, NaCl and phosphate) added to the system, the selectivity of the system was not satisfactory. The lipids and IgY always partitioned in the same phase.

5. Phosphate: Triton system

This biphasic system was discovered when different concentrations of Triton were included in a PEG:phosphate system. For a "pure" system (Triton, phosphate and water) the limits of this system were observed to be 6% for phosphate and 5% for Triton. Triton formed the top-phase. A biphasic system was not obtained below a pH of 6.25 ($HPO_4^{2-}/H_2PO_4^-$ mass-ratio below 0.81).

NaCl had to be added to get a biphasic system when egg yolk was added. With high concentrations of NaCl (over 12%) a whiter top-phase formed consisting of precipitated proteins, including IgY. With 5% egg yolk, a biphasic system resulted at 7.5% phosphate, NaCl and Triton. The system gave a clear, yellow top-phase and a cloudy, white bottom-phase. With centrifugation, a top-phase without a large loss of IgY could be obtained with egg yolk concentration up to 20%. No difference in phase-separation was seen between 21 and 28°C. Phases did not separate as well below 21°C and no phase separation was accomplished below 8°C. IgY losses increased in the top-phase above 28°C.

Attempts made with Tween 20 and Triton X-114 were not successful. Triton X-100 was therefore used for the evaluation and optimization of the system. This system gave the wanted selectivity where the lipids transitioned into the Triton-phase (top-phase) and IgY into the phosphate-phase (bottom-phase).

The experiments showed that the wanted system occurred in a rather narrow zone. With too low a concentration of any component (except egg yolk), a monophasic system resulted. If the concentration of phosphate, NaCl or Triton was too high some

of the IgY precipitated and gave a loss in the top-phase. The optimization was thus aimed at finding concentrations that gave a biphasic system while keeping the loss in the top-phase to a minimum.

Since the experiments indicated roughly the same $HPO_4^{2-}/H_2PO_4^-$ mass-ratio, the factor was taken out for RSM and set to 1.08. Thus only three factors had to be varied, giving star points with a smaller spread. The response variable used was (total amount of IgY)/(amount of IgY in top-phase), with the exception for monophasic systems which were given the grade zero. The desired outcome was that the loss in the top-phase should be small, giving a high value for the response variable.

Ten of the seventeen systems were good, with slight variations. The loss in the top-phase gave a response variable ranging from 0 to 70.25. Differentiation of the second-order polynomial gave optimum at 8.08% phosphate, 9.83% Triton and 7.38% NaCl. The derived levels for the factors gave the desired system, a clear yellow top-phase and a cloudy bottom-phase. This indicated that either optimum or a satisfactory saddle-point was obtained from the differentiation in RSM. The $HPO_4^{2-}/H_2PO_4^-$ mass-ratio in the final "optimized" system corresponded to a pH of 6.32 for the phosphate and water solution.

From the "optimized" system (according to RSM) the yield was calculated to 98.3%. The experiment was repeated for 4 different batches of egg yolk (all from hens immunized against hemoglobin) with a relative standard deviation of 0.15% for the yield. The triglyceride content of the bottom-phase was 22.9% of the total system. The total protein concentration in the top-phase could not be determined since the high Triton-concentration was incompatible with the total protein tests (A_{280} and Bradford). The concentration in the bottom-phase was determined to approximately 50mg/ml with a Bradford test. This corresponded to 150mg/g egg yolk, which was about 95% of the total protein content of egg yolk.

The "optimized" system was also tested for different volumes. No difference in yield, triglyceride content in bottom-phase or IgY-purity in bottom-phase was seen for total system volumes ranging from 2 to 40 ml. This was a positive indication since no changes should therefore have to be made when going to larger centrifugation bottles for use on a larger scale.

The system "optimized" according to RSM seemed to be rather robust. No variations in relative phase volumes or in the color of the phases could be seen for 4 experiments. The high yield was reproducible which could be seen from the low relative standard deviation for the experiments (0.15%).

In conclusion, the phosphate:Triton system provided a very good method as a primary extraction step because of the high yield that could be achieved. The major drawback of the method for use in a large scale production of IgY was the necessity of centrifugation.

The limits of the system were rather sharp, below certain concentrations the result was a monophasic system and above certain concentrations the result was a precipitate in the top-phase.

The phosphate:Triton system was robust with respect to yield within the experimental range set in the robustness test. The operational range for a phosphate:Triton system, giving yields above 97% were 7.8%–8.2% for phosphate, 1.05–1.16 for

HPO_4^{2-}/H_2PO_4 mass-ratio, 9.8%–10.0% for Triton, 7.4%–7.6% for NaCl and 16.6%-16.8% for egg yolk.

The yield of IgY in the phosphate:Triton system was very good but the separation from lipids could be better. The use of CCD was attempted to remove more of the lipids without any significant loss of IgY. A new biphasic system was obtained with 58% primary bottom-phase, 10% Triton and 1.2% phosphate (equal proportions of HPO_4^{2-} and $H_2PO_4^-$). The secondary system was similar to the primary. In the secondary system, the results for yield of IgY and transfer of lipids into top-phase were comparable to the primary system. A 95% yield of IgY in the secondary bottom-phase was obtained with a triglyceride content in the bottom-phase of 23%.

The loss of IgY in a secondary system was the same as in the first system, hence the yield of IgY could still be kept very high after a second cycle. Since the extraction of lipids in the second step was the same, the amount of lipids in the bottom-phase after the second cycle was reduced to about 6.5% of that contained in the total system (lipid content in second bottom-phase was roughly 23% of that in the first bottom-phase, which was about 23% of the lipid content in egg). CCD could therefore be used successfully to further reduce the amount of lipids, while keeping a high yield of IgY. Draw-backs of the phosphate:Triton system was that the system had to be centrifuged to give a good biphasic system.

DISCUSSION

An ATPS is formed when two water-based solutions give a biphasic solution when mixed. The phenomenon of phase separation is disputed. One ATPS theory is based on thermodynamics and states that two factors determine the free energy of mixing of two substances. One is the entropy gain and the other is the interaction between molecules [13]. The gain in entropy is related to the number of molecules involved in mixing and the larger volume accessible to one polymer after mixing. The interaction-energy between molecules increases with the size of the molecules, since it is the sum of the interaction-energy between each small segment of the molecules. This means that for aqueous solutions of polymers, the interaction-energy per mole dominates over the entropy of mixing per mole [3] and phase separation is favoured. The thermodynamic model focuses on the polymers. It does not consider that the system is primarily composed of water. In the lattice model, the solvent (water) plays a key role in explaining phase separation. In solution, a highly ordered layer of hydration surrounds the polymers. Two chemically distinct polymers will give rise to two different hydration layers [13]. If the water structures are incompatible they will give rise to phase separation. The lattice model somewhat explains the cases when biphasic systems do not form. For chemically similar polymers the layers of hydration formed will be similar. Hence, the repulsive effect between the two will not be large enough to induce phase separation.

The most common and well-known system is the one formed from a mixture of Dextran and PEG [2]. In these systems, the top-phase is enriched in PEG and the bottom-phase is enriched in Dextran. The concentration of polymers in each phase depends on the total concentrations of polymers in the whole system. The composi-

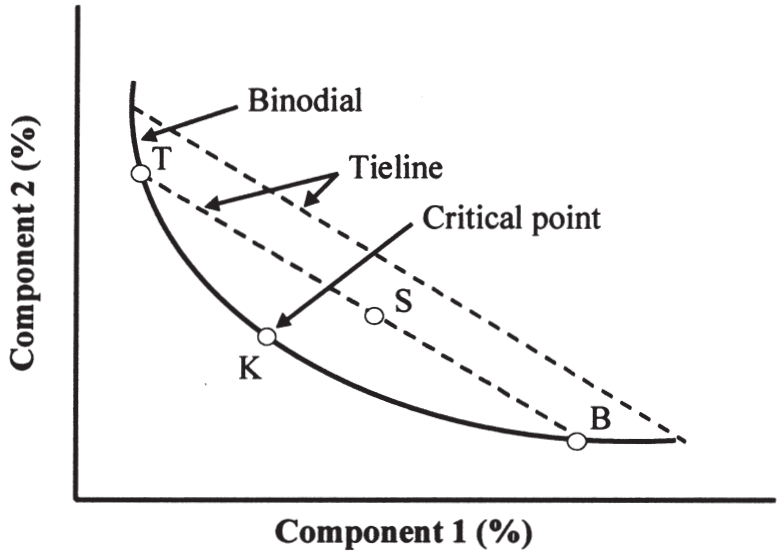

Fig. 1. Phase diagram from mixture of two arbitrary components. S indicates the composition of the mixture. After phase separation, T and B correspond to the composition of the top-phase and bottom-phase, respectively.

tions of the phases in different systems can be determined experimentally and a phase diagram can be constructed. From the phase diagram, the composition of the phases for any mixture within the two-phase region can be predicted [4]. A phase diagram for a mixture of two arbitrary components is shown in figure 1.

The curved line (the binodial curve) separates two regions of compositions. Compositions above the binodial give a two-phase system, whereas compositions on or below the binodial give a one-phase system. An arbitrary composition of the total system within the two-phase region, indicated by S in the figure, will spontaneously separate into two phases with compositions indicated by T (top-phase) and B (bottom-phase). The line connecting TSB is called a tieline. A tieline defines all the different compositions of the total system that give the same compositions in the top-phase and bottom-phase. When moving along the line TSB the only thing changing is the relative volume of the phases. For example, when point S is moved along TSB, away from point B, the volume of the top-phase increases and the volume of the bottom-phase decreases, but the composition of the two remains constant.

ATPS is very easy to scale up. No major changes have to be made when going to larger vessels. Aqueous two-phase systems on a scale up to 1000 kg biomass have been reported for the isolation of target proteins from cell debris and cell culture media [25]. ATPS is thus well suited for large scale purification of yolk antibodies. This in combination with the non-toxic components in the system makes ATPS well suited for the production of yolk antibodies for peroral immunotherapy. One problem with ATPS is that it has to be optimised. We have thus studied the use of ATPS for purification of yolk antibodies. The system described in this paper gives a very high yield of antibodies while removing most of the lipids. ATPS could be used as

an initial purification step. The antibodies recovered from the system can then be applied to an affinity-column without clogging of the column.

Affinity interactions in ATPS have been achieved by coupling biospecific dyes to one of the phase-forming components [15], and by coupling copper (II) iminodiacetate complexes to PEG [9]. The PEG-IDA-metal ion complex primarily increases the partition coefficient for proteins with large amounts of histidine residues [6]. In a system where affinity purification is wanted, the ligand is preferably coupled to PEG [17]. Coupling of various proteins have successfully changed the partition coefficient when, for example, isolating cells [10], membranes [18] and proteins [11].

When PEG was added to a phosphate:Triton system, a third clear phase was formed between the top-phase and bottom-phase. This was assumed to arise from PEG forming a separate phase. The phase was shown to be depleted of proteins by SDS-PAGE. The volume of this third phase increased with an increase in amount of PEG added. Additions of PEG to a concentration in the total system of 1%–3% gave the same results, except that the volume of the PEG-phase increased. The three-phase system obtained when PEG was added did not facilitate the phosphate:Triton system. However, it might give a possibility of isolation of antigen specific IgY. If PEG were coupled with the antigen, the specific antibodies would probably be separated into the PEG-phase, which is depleted of other materials. The system would extract lipids into the Triton-phase, specific IgY into the PEG-phase and all other proteins into the phosphate-phase. Since no affinity chromatography would be needed, the production-cycle could be greatly decreased. This type of system would be interesting to test in the future.

In conclusion, ATPS is a new method for the purification of yolk antibodies which is well suited for large scale purification of antibodies. The phosphate:Triton system provides a very good method as a primary extraction step because of the high yield that can be achieved. The drawback of the method for use in a large scale production of IgY is the necessity of centrifugation.

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