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Coupling purification and in situ immobilization process of monoclonal antibodies to clenbuterol for immunosensor application

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ABSTRACT

Clenbuterol (CL), which promotes the growth of muscular tissue and the reduction of body fat in pigs and cattle, has been confirmed to be a potential hazard to human health. In this study, a monoclonal antibody to clenbuterol (CL mAb) from a hybridoma culture supernatant was purified by an aqueous two-phase system (ATPS) at different polyethylene glycol (PEG) concentrations, PEG molecular weights, pH values, and NaCl concentrations. Then the CL mAb was immobilized in situ by directly adding polystyrene microspheres (PSMSs) into a PEG phase containing CL mAb. Using the immobilized antibody, an immunosensor was constructed to detect the CL residues in pork samples. The results showed that using an ATPS composed of 15% (w/w) PEG6000, 15% (w/w) phosphate, and 15% (w/w) NaCl at pH 8.0, the partition coefficient was 7.24, the activity recovery was 87.86%, and the purification fold was 2.88. The PEG–CL mAb–PSMS retained approximately 98% of its initial activity after 30-ml phosphate buffer (PBS) washings. After 30 days of storage, the CL mAb–PSMS lost nearly 75% of its activity, whereas the PEG–CL mAb–PSMS retained as much as 95% of its initial activity. Furthermore, the constructed immunosensor obtained recoveries of 90.5 to 102.6% when applied to pork samples spiked with CL.

Clenbuterol (CL)[2] is a β-adrenergic drug usually employed as a bronchial dilating agent for the treatment of pulmonary diseases in humans and animals [1]. Due to its growth-promoting effect involved in increasing lean muscle mass and reducing fat deposition, CL is also commonly but illegally added at high doses to livestock feed, especially for pigs and cattle, to improve the production of lean meat [2]. Intake of CL may result in human food poisoning, including muscular tremors, tachycardia, palpitation, and dizziness; hence, its use has been banned in livestock feed in most countries [3]. Although China has taken measures to restrict the use of CL in livestock feed, CL poisoning still occurs frequently.

A number of analytical methods have been reported for the detection of CL. Immunoassay methods based on the specific recognition of antigens by antibodies have received greater attention, especially when used as immunosensors [4]. In the immunosensor method for detection of residues, antibodies are usually immobilized as probes onto different solid surfaces by physical adsorption or covalent coupling. Immobilization allows for increased antibody–antigen binding to improve detection sensitivity [5–7]. Although the immunosensor method has been employed as a rapid, efficient, and convenient detection method for pollution residues, the commercialization of immunosensor technology has achieved only limited success, mostly because of the generation of false signals caused by instability and poor loading of the immobilized antibody. To solve these problems, many processes have been proposed for antibody immobilization. Some of the most common methods, such as entrapment and physical adsorption, generally suffer from antibody leaching and/or diffusion limitations during operation. On the other hand, immobilized antibodies prepared by covalent bonding are frequently inactivated during the immobilization processes. Thus, high stability and bioactivity of the immobilized antibodies with simple immobilization processes are typically required to reduce false signals in the immunosensor.

To obtain increased antibody loading and activity, Guan and coworkers proposed that mouse ascites, hybridoma culture supernatants, and genetic engineering products containing monoclonal antibody (mAb) need to be purified before immobilization [8]. However, the traditional methods reported for mAb purification, mainly chromatography-based technologies, are too expensive

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[2] *Abbreviations used:* CL, clenbuterol; mAb, monoclonal antibody; ATPS, aqueous two-phase system; PEG, polyethylene glycol; HRP, horseradish peroxidase; IgG, immunoglobulin G; TMB, 3,3′,5,5′-tetramethylbenzidine; PSMS, polystyrene microsphere; NaCl, sodium chloride; PBS-T, phosphate buffer containing Tween 20; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AFM, atomic force microscopy; ELISA, enzyme-linked immunosorbent assay; FIA, flow injection analysis; RSD, relative standard deviation; M_W, molecular weight.

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and complex to scale up production for increasing mAb demands. Recently, some separating technologies with simplified process were introduced [9–12], showing the aqueous two-phase system (ATPS) as an ideal purification technique for the extraction and concentration of biomolecules because of its high productivity, simplicity, short processing time, cost-effectiveness, scalability, and versatility. An ATPS is usually formed by combining either two incompatible polymers such as polyethylene glycol (PEG) and dextran or a polymer and a salt, commonly phosphate [12]. Compared with the PEG–dextran system, the PEG–phosphate system can efficiently separate proteins with different hydrophobic properties because of the phase hydrophobicity and salting-out effects. Moreover, the PEG–phosphate system is easier to operate and scale up due to lower viscosity and lower cost of the salt phase [13]. ATPS technology has successfully been applied to the separation and purification of biological products such as proteins, nucleic acids, and viruses [14–16]. However, after purification by ATPS, some technical difficulties remain, including recovery of antibodies dispersed in the PEG phase and recycling of the PEG.

Schulze and Winter and others reported that when native macromolecules were incubated in PEG, almost no loss of activity was found [8,17]. Bradoo and coworkers recovered enzymes from the phosphate phase of an ATPS by immobilizing directly on solid carriers, thereby allowing the recycling of the bottom salt phase [18]. Based on these reports, an alternative process that includes the purification of CL mAb by an ATPS and its in situ immobilization directly in the PEG phase was hereby explored. Evidently, this novelty method could allow antibody recovery and immobilization in the PEG phase simultaneously, avoid the use of other complicated steps, and allow the recycling of the top PEG phase.

In current work, the objective was to investigate an effective approach for the purification and in situ immobilization of CL mAb in an ATPS system and apply the immobilized mAb in immunosensors for detecting CL in pork samples. A schematic diagram of the experimental procedure is shown in Fig. 1.

Materials and methods

Materials

Hybridoma cells secreting CL mAb were cultured, and the cultured supernatant containing CL mAb was centrifuged and collected for later purification. Horseradish peroxidase (HRP)-labeled CL was prepared using EDC/NHS [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide] crosslinking, as described by Roda and coworkers, with a coupling ratio of 1.8 [19]. PEG with molecular weights of 1000, 2000, 4000, 6000, 10,000, and 20,000 Da were purchased from Fluka (Buchs, Switzerland) and used without further purification. HRP-conjugated goat anti-mouse immunoglobulin G (IgG) was provided by Agrisera (Vännäs, Sweden). 3,3′,5,5′-Tetramethylbenzidine (TMB) was obtained from Sigma–Aldrich. Polystyrene microspheres (PSMSs) of 3 mm diameter were produced by Janus Materials (Nanjing, China). All other chemicals were of reagent grade or higher.

ATPS extraction and characteristics of CL mAb

ATPS extraction of CL mAb

Aqueous PEG–phosphate systems were prepared by weighing appropriate amounts of PEG stock solution (40%, w/w), phosphate buffer stock solution (40%, w/w), sodium chloride (NaCl) solution, hybridoma culture supernatant containing CL mAb, and water to a final weight of 10 g. Phosphate buffer solutions (40%, w/w) with different pH values were prepared by using variable mass ratios of K_2HPO_4 to $N_aH_2PO_4$. Slight adjustments to the final pH value were performed with a 40% (w/w) solution of K_2HPO_4.

All system components were thoroughly mixed on a vortex shaker, equilibrated at 4 °C for 60 min, and then centrifuged at 1500*g* for 10 min to ensure separation into two phases. The top and bottom phases were then carefully separated, and the volume of each phase was measured. Samples were taken from the hybridoma culture supernatant, the top phase, and the bottom phase of the system for determining protein concentration and CL mAb activity. The partition of CL mAb was described by the partition coefficient (K), the activity recovery of CL mAb in the top phase (Y), and the purification fold (PF).

The partition coefficient (K) of CL mAb was defined as the ratio of total activity in the top phase (A_t) to that in the bottom phase (A_b). It was calculated as follows:

$$K = \frac{A_t}{A_b}.$$

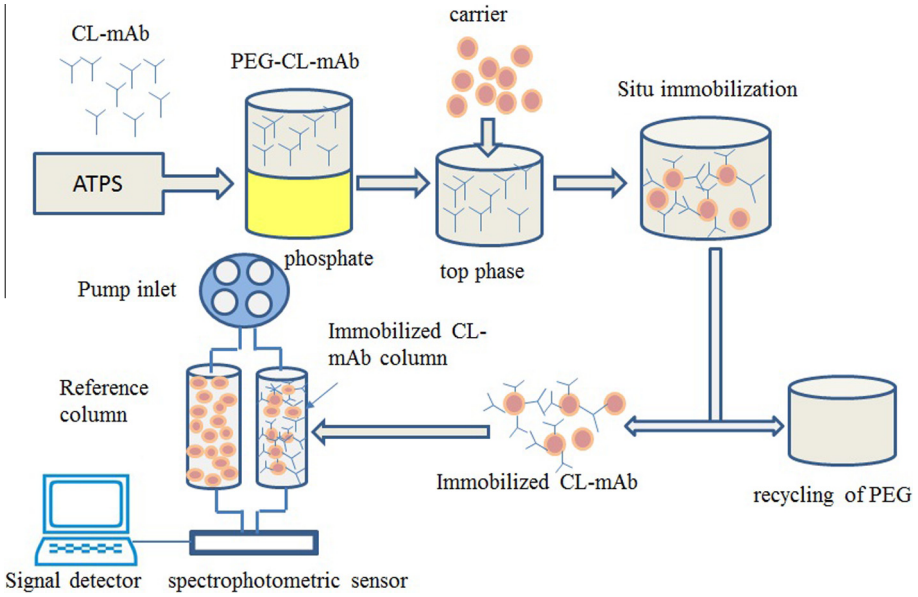

Fig.1. Schematic diagram of the experimental procedure.