1. Introduction

Thalassemias are a group of inherited hemoglobin (Hb) synthesis disorders characterized by the reduction or absence of one or more of the globin chains of Hb [1-3]. This deficiency leads to imbalanced globin chain synthesis and accumulation of excess γ-globin and β-globin chains in α-thalassemia and to elevation of Hb Aβ levels with varying levels of Hb F in β-thalassemia [1-7]. At present, thalassemias have become a global problem, not only as a health problem but also as a socioeconomic problem [7,8]. In Thailand, both α- and β-thalassemias are prevalent. Approximately 20% to 30% of the population are α-thalassemia carriers, and 3% to 9% are β-thalassemia carriers [7,9]. α-Thalassemia is characterized by a decrease in or an absence of α-globin chain production, which gives rise to α-thalassemia 2 (or α-α-thalassemia) and α-thalassemia 1 (or αβ-thalassemia), respectively [1-4,10]. β-Thalassemia results from a reduction in or an absence of β-globin chain synthesis, leading to β'-thalassemia and ββ'-thalassemia, respectively [2-4,10]. Hb Constant Spring (CS), an elongated α-globin chain, also has an α-thalassemic effect. Interaction
between α-thalassemia 1 and α-thalassemia 2 or Hb CS leads to the same phenotype, which is called Hb H disease. Because Hb E (αβθ,1626Glu→Lys) also has a β-thalassemic effect, interaction between Hb E and β-thalassemia genes results in β-thalassemia/Hb E disease, which can be as severe as homozygous β-thalassemia. Different combinations of abnormal genes may lead to other complex thalassemia syndromes, such as EA Bart’s and EF Bart’s diseases [10].

Many methods are currently used for the screening and diagnosis of thalassemias. The osmotic fragility test is commonly used for screening thalassemias, but its specificity is low [2,4-6]. The most precise method for thalassemia diagnosis is phenotyping by Hb electrophoresis or high-performance liquid chromatography (HPLC) and genotyping using the polymerase chain reaction [2,4-6,10-12]. However, these techniques are sophisticated and cannot be used as routine laboratory tests in small hospitals or clinics. They are, therefore, not practical for rapid population screening. Recently, various types of immunoassays have been developed and widely applied for the screening or diagnosis of various diseases. The immunologic technique is simple and inexpensive with high sensitivity and specificity. Furthermore, a large number of samples can be analyzed in a short time. Therefore, this technique should be appropriate for screening and diagnosis of thalassemias in resource-limited countries.

Immunoglobulin Y (IgY), the predominant serum immunoglobulins in birds, reptiles, and amphibians, is transferred from the maternal serum to the egg yolk to confer passive immunity to embryos and neonates [13-15]. In the chicken, it is very easy to induce a humoral immune response, and the induced antibodies are passed to the egg yolk in a high quantity. Approximately 75 to 100 mg of IgY can be obtained from a single egg, and up to 1500 mg of IgY can be harvested from eggs every month [16,17]. IgY antibodies are easy to isolate from the egg, and the collection of blood, which is painful for the animal, is not required. In addition, the chicken has a better immune responsiveness to mammalian antigens because of its phylogenetic distance. This quality makes the production of antibodies against conserved mammalian proteins more successful in the chicken than in mammals. Because of these advantages, the production of IgY antibodies from chicken egg yolk, termed IgY technology, has been developed to produce polyclonal antibodies against various proteins of interest [18-20]. In an attempt to develop an immunoassay for the diagnosis of thalassemia, we applied IgY technology in the present study to the production of polyclonal antibodies against Hb Bart’s. The antibodies produced were then used to establish an enzyme-linked immunosorbent assay (ELISA) for thalassemia diagnosis.

2. Materials and Methods

2.1. Hemolysate Preparation

Informed consent was obtained according to the guidelines of the human experimental committee of Mahidol University. Blood samples were collected from thalassemic patients, healthy subjects, normal umbilical cord, and stillborn infants with Hb Bart’s hydrops fetalis by using EDTA as anticoagulant. Hematologic data were determined with an automated cell counter (Advia 120; Bayer HealthCare, Tarrytown, NY, USA). Hb typing was performed with a fully automated HPLC system (Variant; Bio-Rad, Hercules, CA, USA) [21]. Plasma was removed, and the red blood cells were washed 3 times with normal saline solution. Then, 1.5 volumes of distilled water and 0.5 volumes of toluene were added to the packed red blood cells, followed by vigorous mixing for 5 minutes and centrifugation at 1200g for 30 minutes. The toluene layer and the red cell stroma were discarded, and hemolysates were kept at –20°C until use. The protein concentration of the hemolysates was determined with the Bradford reagent (Bio-Rad). The phenotypes of the hemolysates from normal cord, healthy adult, and stillborn Hb Bart’s hydrops fetalis samples were reconfirmed by isoelectric focusing using a Hemoglobin Test Kit (PerkinElmer Life and Analytical Sciences, Walton, OH, USA) and by cellulose acetate electrophoresis [22].

2.2. Purification of Hbs and Globin Chains

Hb Bart’s (γδ) was purified from Hb Bart’s hydrops fetalis hemolysate by diethylaminoethyl (DEAE)-cellulose chromatography on a 1.8 cm × 20 cm column with 0.2 M glycine, 0.1 g/L potassium cyanide buffer, and a sodium chloride gradient, as previously described [22,23]. The hemolysate was dialyzed against distilled water at 4°C overnight before it was loaded onto the column. The absorbance of the effluent fractions was measured at 415 nm, and the ionic gradient was followed by measuring the conductivity. After chromatographic separation, 6 effluent fractions were obtained. Because Hb Bart’s, Hb Portland, and other Hb components were not completely separated, fractions that contained primarily Hb Bart’s were pooled and concentrated with a Millipore Filtration Unit (Millipore, Bedford, MA, USA). The concentrated solution was dialyzed against water, lyophilized, and further purified by anion-exchange HPLC on a nonporous DEAE column (SPW, 7.5 mm × 7.5 cm; TSKgel, 2.5 μM) (Tosoh, Tokyo, Japan) [24].

DEAE-cellulose chromatography was also used to purify Hb A and Hb A2 from hemolysates from healthy adults, as well as Hb F from normal cord hemolysates. For the isolation of globin chains, Hbs were first subjected to acid acetone precipitation [25,26] and then fractionated by reversed-phase HPLC on a 1 cm × 25 cm Protein C semipreparative column (Grace Vydac, Hesperia, CA, USA) by using developer with a gradient of solution A (acetonitrile-water [60:40] containing 0.1% trifluoroacetic acid) to solution B (acetonitrile-water [20:80] containing 0.1% trifluoroacetic acid) [26]. All Hbs and globins obtained were checked for purity by Triton X-100–acetic acid–urea polyacrylamide gel electrophoresis (PAGE) [27], lyophilized, and stored at –20°C until use.

2.3. Chicken Immunization

A 5-month-old egg-laying Rosehorn chicken was kept in an individual cage with ample food and water ad libitum throughout the course of the study. For immunization, lyophilized Hb Bart’s was dissolved to 1 mg/mL in phosphate-buffered saline (PBS). At day 0, 500 μg Hb Bart’s in complete Freund’s adjuvant (Sigma-Aldrich Corporation, St. Louis, MO, USA) was injected at 2 sites via the intraperitoneal muscle. Boosters of 500 μg antigen in incomplete Freund’s