

Increased the specificity and sensitivity of monospecific antibody against host cell protein (HCP) in quality control of hepatitis B recombinant vaccine

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ABSTRACT

One of the most important aspects in recombinant biologic production, based on GMP rules, is the accuracy of final product quality control, especially assessment of host cell macromolecules contamination rate in final product. The purification requirement can be eliminated when the yeast cell containing the recombinant protein is used as a host cell. It is possible that the final product contaminated to the host cell protein during purification stages of HBsAg (HBV vaccine). The protein purification costs depend on the purification procedures required. Nowadays several companies produce commercial kits for identification and assessment of host cell protein contamination based on ELISA and Western blotting methods. But high prices, difference in sensitivity and lack of easy access to these kits sometimes create problems. So, in this study, two methods of Ammonium sulphate and caprylic acid precipitation technique were used separately for IgG purification. The results showed that IgG purification increased by 97% in caprylic acid method, compared with only a 77% increase in ammonium sulphate method. There were also significant differences in specificity and sensitivity between our standardized ELISA technique and using commercial kit (Cygnus CHO HCP).

Key words: Host Cell Protein (HCP), Recombinant hepatitis B virus (HBV) vaccine, Contamination.

INTRODUCTION

Hepatitis B virus is one of several hepatitis viruses that cause a systemic infection, with a major pathology in the liver. These include hepatitis A virus, hepatitis B virus, and hepatitis C and E viruses, previously referred to as non-A, non-B hepatitis viruses. Hepatitis B virus is an important cause of viral hepatitis. Effective and safe hepatitis B virus vaccine was commercially available since 1982[1]. First vaccines were prepared by harvesting HBs Ag from the plasma of patients with chronic HBV infection. Then with the development of recombinant DNA technology, HBsAg was expressed in other organisms. Today, recombinant DNA vaccines are used in most countries [2, 3]. Hepatitis B virus vaccine contains 3 to 40 micrograms per liter of HBsAg protein and ammonium phosphate or aluminum hydroxide adjuvant

[1]. The most widespread recombinant hepatitis B vaccine is a hepatitis B surface antigen (HBsAg) with 226 amino acids expressed in yeast *Saccharomyces cerevisiae* and *Pichia pastoris* [4,5] are used for preparation of recombinant hepatitis B vaccine [13], and due to advantages of *Pichia pastoris* over *Saccharomyces cerevisiae*, this yeast is used most [13,14]. HBsAg produced in yeast, is purified by physical separation techniques, then yeast components are removed. There is possibility of contamination of final product to the host cell protein (yeast) in HBsAg purification stages [1, 8]. All yeast, plant and animal-derived recombinant proteins that are administered to human patients must be compared to their counterparts and rigorously tested for safety [5, 6]. Quality control of using materials, intermediate products and finally the finished product at different stages of vaccine production process, are the most important steps in the

GMP principles. One of quality control tests in the production of recombinant hepatitis B vaccine is determining the final product contamination of host cell proteins (HCP) [7, 8]. According to WHO protocol and valid pharmacopoeia, this amount should not exceed 0.1 Percent [9]. Since for production of recombinant products, especially hepatitis B vaccines, the recombinant DNA of host cells are used, even after the purification process, some impurities remains in these products. Although the infection rate may be low, but for protection of consumer health and prevention of immune

response, contamination level should be decreased to the minimum amount (less than 0.1%). Commercial reagents and generic analytical methods are available to quantify most of these contaminants that each of them possess advantages and disadvantages (Table 1) [10, 11]. Two current Immunological methods that use to assess the HCP contamination are Western blot and immunoassay [19]. Western blot is used to confirm the presence of infection (qualitative), while immunoassay is effective for determination of the contamination rate (quantitative) [7, 12].

Table1: comparison of purification methods.

Method	Advantages	Limitation
SDS-PAGE/ Silver stain	Good sensitivity 100pg/band, resolves, multiple Components	Subjective, interpretation qualitative, complex, and technique-dependent
HPLC	High resolution	Low sensitivity, nonspecific, Quantitative
Western blot	Immunological identity resolves multiple components sensitivity 0.1–1 ng/band	Qualitative, very complex antibody may fail to detect some contaminants
Immunoassay	High sensitivity ng/mL quantitative, objective endpoint	No resolution of individual components, antibody may fail to detect some Contaminants

Nowadays there are commercial kits that recognize host cell proteins by using polyclonal antibodies [8,11]. In general, in biological production, including pharmaceutical, food or products that are used in diagnostic kits, purification and filtration are the most expensive stages, so it seems that in commercial kits for detection of recombinant hepatitis B vaccine pollutant proteins, rabbit blood hyper immune total serum is used in order to decrease production costs [4, 13]. Therefore use of purified and mono-specific antibodies can significantly increase results reliability, because in comparison with commercial kits sensitivity and specificity are better [15, 17]. The present research has been done at Pasteur Institute of Iran to increase the sensitivity and specificity of HCP contamination detection test.

MATERIALS & METHODS

Yeast strains

Recombinant *Pichia pastoris* (W-3160) and non-transgenic *Saccharomyces cerevisiae* (CSBPI-d-41) (as negative control) were obtained from microbial strains maintenance center of Pasteur Institute of Iran.

Preparation of specific *Pichia pastoris* antiserum

In order to prepare *Pichia pastoris* lysate for injection, yeast was placed in YPG broth medium (1% yeast extract, 2% peptone and 2% glucose) at 30°C on the shaker for 72 hours [15,17]. Then 0.5 mm of glass seeds was added and spin at 2 to 5°C. Yeast lysate was administered by subcutaneously injection. So, 0.5ml of cell lysate was emulsified with complete Freund's Adjuvant and was injected into white albino rabbits. This procedure was repeated twice, after 14 and 28 days after the first immunization. After one week, the animals were bled and blood serum was maintained at -20°C [5, 19, 20].

Separation of Immunoglobulin

Two methods were used:

A) Antibody purification using ammonium sulfate precipitation:

10 ml of saturated of ammonium sulfate (100 g ammonium sulfate powder in 100 ml distilled water) was added to 20 ml of prepared serum. This solution was centrifuged for 10 minute at 1000 rpm. The supernatant discarded and precipitation was solved with 5 ml phosphate buffer with pH = 7 (16 g sodium chloride, 4 g

potassium chloride, 23 g di-sodium hydrogen phosphate and 4 g potassium dihydrogen phosphate) [5, 11, 22, 23].

B) Antibody purification using Caprylic acid precipitation:

In terms of yield of antibodies, precipitation with caprylic acid is comparable to sequential precipitation with ammonium sulfate method. In this method, 10 ml of prepared serum was added to 30 ml of 60mM sodium acetate and pH adjusted to 4.5 to 4.8 with HCl. 700 micro liter caprylic acid was added gently and solution was mixed by magnetic mixer at 25°C for 1 hour. Then supernatant containing antibody was isolated by centrifuge at 5000 rpm for 15 minutes [23,24].

Ion Exchange Chromatography

Total prepared protein was used for DEAE-cellulose ion exchange chromatography. Chromatography was equilibrated with phosphate buffered saline pH=6.3. Then sample was used as feed and bound antibody was either eluted using phosphate buffered saline pH=6.3. Other attached proteins were separated by sodium chloride 1M [4, 6, 10, 22].

SDS-PAGE electrophoresis

Purified IgG was boiled in buffer sample without 2ME for 5 minutes. Also to confirm and further review a sample, purified IgG was boiled in buffer sample with 2ME. These samples separately were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 45 minutes at 14 mA, followed by electrophoretic transfer to nitrocellulose membrane [6, 22].

Immunoblotting test

For Western blotting, after protein transferring to nitrocellulose membranes, membranes were blocked and incubated with BSA 5% for 30 minutes. After washing with phosphate buffered saline pH=7.2, membrane was incubated with appropriate primary antibodies (purified IgG) at room temperature for 1 hour, followed by incubation with anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies. The probed proteins were detected using the chemiluminescent reagent. The bidimensional absorbance of proteins on the films were quantified and analyzed with Molecular Analyst

software (Bio-Rad). In this blotting, control antibody was Pichia pastoris standard antibody [5, 12, 27].

ELISA

ELISA was performed with the samples taken before, during and after purification in order to determine the concentration of the protein at stages of purification. Polyclonal affinity purified anti pichia pastoris antibody was used for coating of ELISA plates. Conjugated IgG antibody was used as the primary antibody. Bound yeast antigen was detected by addition of peroxidase-conjugated anti-yeast antibody. ELISA readings (OD 450 nm) were performed after 1-10 min for detection of peroxidase [4,6,13,23,27].

Statistical analysis

Quantitative comparisons of immunoblotting variables between purified antibody and kit were examined with the PRISM3 test and t test ($p < 0.0001$). Continuous variables were dichotomized to facilitate our analysis [13, 27, 23].

RESULT

IgG antibody purity was tested at several stages during purification using SDS-PAGE electrophoresis, and quantified the increased binding affinity. Purification of rabbit IgG specific antibodies by precipitation with caprylic acid was compared to that 1) by sequential precipitation with ammonium sulfate 2) by Cygnus CHO HCP ELISA kit. In terms of purity of antibodies, precipitation with caprylic acid (99%) is more efficient than the ammonium sulfate method (77%). It should also be noted that precipitation with caprylic acid is associated with an increase in the affinity of some antibodies and is suitable to purify rabbit IgG. Electrophoretically separated proteins were transferred from a SDS-PAGE to a nitrocellulose membrane in the presence of transfer buffer. In addition, ELISA results confirmed that elution of IgG from the cation exchange matrix was also a very important step to optimize. The ELISA results were also confirmed by immunoblot analysis of the IgG purification. ELISA test was performed to

measure the rate of contamination of hepatitis B vaccine by *Pichia pastoris* proteins, using purified and mono-specific antibody on 23 samples three times (table2). Also Pasteur

Institute of Iran, Hepatitis B vaccine contamination rate was measured by Cygnus CHO HCP ELISA kit.

Table2: Specificity and sensitivity comparison of purified mono-specific antibody from chromatography by ELISA and Cygnus CHO HCP ELISA kit.

Sample HBV vaccine	Mono-specific Ab	Kit	Sample HBV vaccine	Mono-specific Ab	kit
1	60.79	26.77	13	50.39	11.13
2	38.09	37.17	14	75.75	54.43
3	48.65	44.97	15	70.15	51.53
4	174.29	151.67	16	109.09	113.27
5	30.52	23.17	17	137.35	105.27
6	37.52	33.97	18	43.52	41.73
7	0.72	0.00	19	61.35	32.10
8	47.55	8.93	20	160.75	124.30
9	64.62	47.33	21	16.19	14.33
10	49.25	44.67	22	71.95	15.63
11	69.85	0.00	23	148.55	80.63
12	65.29	28.90			

Sensitivity and specificity of ELISA was performed for both antibodies using the following formula

$$\text{Sensitivity} = \frac{TP}{TP+FN}$$

$$\text{Specificity} = \frac{TN}{TN+FP}$$

FP= False Positive

TP= True Positive

FN= False Negative

TN= True Negative

The results indicate that the sensitivity and specificity of produced antibody is higher than commercial kit (table3).

Table 3. Increased sensitivity and specificity of purified mono-specific antibody in comparison to kit.

Kit	Mono-specific Ab	It
0	0	FP
23	23	TP
2	0	FN
1	1	TN

DISCUSSION

Contamination of intravenous solutions with unwanted macromolecules can cause allergy and numerous adverse reactions in human, so, quality control of these products is very

important [1,27,28]. Identification and assessment of host cell protein contamination in hepatitis B vaccine has important role in quality control tests [7]. According to World Health Organization, contamination rate

should not be more than 0.1 micrograms per dose of vaccine [9]. Confidence in the quantitative nature of a given HCP assay, and the validity of analytical measurement obtained by the assay, is dependent upon empirical demonstration of the unique stoichiometry of the HCP assay reagents. In conjunction with other analytical and validation methods, an HCP immunoassay may be thought of as a necessary quantitative tool for the optimization and validation of biopharmaceutical purification process efficiency and consistency, rather than as an end in itself. Production batches of the Hepatitis B vaccine should be tested by the National Control Laboratory (NCL) before being released to the market.

In production of biological products that use in diagnostic kits, purification has the highest production cost. Now several companies produce several commercial kits for identification and assessment of host cell protein contamination based on ELISA and Western blot methods [4, 8, 10]. But high prices, commercial quality, difference in sensitivity and lack of easy access to these kits, sometimes create problems. Commercial enzyme-linked immunosorbent assay (ELISA) kits for the determination of contaminant have been used frequently as an important method for tests. With the constant need for validation procedures, an ELISA that could be employed to determine the recombinant hepatitis B vaccines was established using mono-specific antibodies.

The development of hepatitis B vaccine was described in yeast cell origin. HBsAg was produced in recombinant yeast cell culture. Now the purified anti *pichia pastoris* antibody in several quality control measures are available to ensure clients are supplied with the highest quality of purified proteins [25]. The results of research conducted by Mohanty JG, Elazhary Y suggests that IgG prepared by ammonium sulfate is better than that by caprylic acid method but the results of this research indicated the superiority of serum proteins purification by caprylic acid method [29]. The present investigation shows that producing standard mono-specific antibodies by laboratories of recombinant vaccine production centers, in addition to lack of need for commercial kits, can test host cell protein

contamination of the vaccine and increases the quality of productions than enter marketing. It can be concluded that production and use of mono-specific antibodies for recombinant products quality control leads to more desirable and cost effective results [12, 13]. Since the recombinant hepatitis B vaccine is an important production in Pasteur Institute of Iran, the needful researches has been done on this product. It is to be mentioned that the achieved results could be forecasted on other recombinant products.

The use of mono-specific antibody produced "in house" specific for HCP of the hepatitis B virus (HBV) vaccine can result in the production of a sensitive, highly specific and stable ELISA [4, 13, 16].

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