Research Article

Journal of Biotechnology and its Applications

Saccharomyces Specific- HCP Assay for r-PAI-2 Therapeutic Proteins

G-Halli Rajasekariah*

Biofirm Pty Ltd 19, Burraneer Avenue, St Ives NSW 2100, Australia.

***Correspondence:**

G-Halli Rajasekariah, Bio firm Pty Ltd 19, Burraneer Avenue, St Ives NSW 2100, Australia, E-mail: grajasekariah@gmail.com.

Received: 02 Feb 2021; **Accepted:** 23 Feb 2022; **Published:** 01 Mar 2022

Citation: Rajasekariah HG. Saccharomyces Specific- HCP Assay for R-PAI-2 Therapeutic Proteins. J Biotechnology App. 2022; 1(2); 1-12.

ABSTRACT

Whenever a host cell is employed for a means of propagation of recombinants, some impurities are bound to come across and they are invariably of host cell components and are part of final recombinant proteins. These are investigated for the first time in case of recombinant host, "yeast cells" Saccharomyces. With great care, high affinity antibodies are generated for yeast proteins using Gene-minus (null-cell) yeast components purified by down-stream techniques with appropriate resins for column purification and these antibodies are being used to construct specific sandwich ELISA system to monitor the degree of host contaminants proteins (HCPs). How this is being done and what strategic methods are followed and how the HCP- detection ELISA systems are constructed and what are the limitations?. These points are described and discussed along with prospects of HCPs in the intensified therapeutic proteins preparation. Our HCP assay is sensitive enough to detect 200-250 ppm in the final PAI-2 product.

Keywords

Saccharomyces, Recombinant proteins, Gene-minus null-cells, Down-stream techniques, Q-Sepharose, Ph-Sepharose, Tentacle ion exchange resin, Column chromatography, High affinity antibodies, Host-contaminant proteins, r-PAI-2, Two site ELISA system.

Introduction

The recombinant DNA technology employs either a prokaryote (eg a bacterium, *E. coli*) or a lower eukaryote (eg a yeast, *Saccharomyces*) as the host cell for inserting "the gene of interest" for production of therapeutic proteins by the fermentation process. Prokaryotes are too far away evolutionarily from higher eukaryotes. *E. coli* organizes, duplicates, and separate their chromosomes by means different from those in eukaryotes; bacteria and eukaryotes also differ in types and function of their sub-cellular organelles; and they differ in metabolism and homeostatic regulation. Molecular genetic studies of eukaryotes require experimental systems closer in evolution than bacteria can provide. They offer the experimental tractability of bacteria along with many basic biological properties of complex eukaryotic cells; the eukaryotic microorganism that has been most highly developed as an experimental system is *S. cerevisiae*, a free-living yeast with excellent classic genetics. *S. cerevisiae* has a more tractable mitochondrial genome. The yeast genome contains the basic blue print of all eukaryotic cells in miniature. In other words, there is roughly 10 times less DNA in their entire yeast genome localised on 16 chromosomes than a single human chromosome. In yeast, coding sequences are compressed into smaller, denser packages interrupted by fewer introns. Several different kinds of yeast vector systems have been developed. The yeast and mammalian proteins are similar enough in amino acid identity to functionally interchangeable [1]. *Saccharomyces* has already begun to play a central role in both the pharmaceutical and industrial arenas. Yeast has several advantages for processes that require production on a large scale: the low cost of culture media and a history of efficient fermentation technology. Yeast has been eschewed for productions of therapeutic proteins because of the complex polysaccharides that decorate proteins secreted from yeast are immunogenic [2]. This eukaryotic expression system provides glycosylated final product with human-like glycan structures unlike *E. coli* bacteria [21,24]. PAI-2 gene sequence (see below) was inserted into a plasmid vector and transformed the yeast (*Saccharomyces*) where it was expressed the targeted PAI-2 therapeutic protein. The expression depends upon the multiplication of yeast cells. In this way, recombinant DNA technology is useful in producing therapeutic proteins of interest. It helped to produce large amounts of therapeutic material at a competitive cost while ensuring that the product quality is maintained [3,21,22].

Besides the fusion product, several other proteins are expected to be released by these host cells due to degradation or due metabolic functions. This forms the major source of host contaminant proteins (HCPs) [4,5]. HCPs are a complex mixture with diverse physicochemical and immunological entities. The physiochemical properties of the intended recombinant protein (charge, hydrophobicity, structure etc) influence the HCPs present at various stages in the bioprocess [6]. A variety of chromatographic analyses is required to purify the product and to achieve homogeneity in the final product. Detection of HCP of contaminants or any impurities in any recombinant DNA product is of vital importance for establishing the purity of the product [4]. This is essential from the Regulatory Authorities point of view. PAI-2, an anti-inflammatory recombinant product manufactured in *Saccharomyces cerevisiae* system at Biotech Aust Pty Ltd. Tracing of 'yeast-HCPs' as impurity in the recombinant PAI-2 therapeutic product forms an essential part of rigid quality control and product assurance procedures. The level of HCPs in therapeutic proteins are most often expressed as nanogram HCPs per milligram of therapeutic proteins (ng/mg), or parts per million (ppm) determined by a quantitative Enzyme-linked-Immunosorbent Assay or Immuno- (Western) Blotting with anti-HCP sera [6]. We report here the technology and methodology followed in the generation of a panel of GM-antibodies against different components of downstream proteins (DSP). This necessitated the generation of high affinity gene minus antibodies (GM-PAbs) for the prevailing components in Gene-minus (null-cells) preparation. As the Gene-minus preparations (null-cells) are a complex set of protein components, some care is essential to monitor the immunised animal for the appropriate immune response against these components. Biotechnology Industry addresses this risk and therefore appropriate level of bioprocess was instituted for detection of any level of impurities (HCPs) in the final recombinant product [8]. That is why down-stream process has become so stringent methodology, which has been applied with great caution and care to minimise the impurities (HCPs).

Materials and Methods

Preparation of Gene-minus recombinant host (yeast) material

Gene-minus *Saccharomyces cerevisiae* strain (free from PAI-2 gene but containing the vector 8630 bp) (null cell) (BTA2310) was subjected to chromatographic analysis and 4 antigenic preparations were obtained. PAI-2 was purified by subjecting the 0.2 µm filtrate through 1^{o}Q-Sepharose, Phenyl-Sepharose and 2^{o}Q-Sepharose. This can be prepared by mimicking the chromatographic purification (mock chromatography) that has been used for the final PAI-2 product. The recombinant host-preparation would serve as a reference and should follow identical steps of purification that has been standardised for the final r-PAI-2 product. The required material was produced by mock fermentations (a 50 L run Fermentation Batch P-163). The Gene-minus material (null cells) was subjected to mid-stream (MSP) and down-stream purification (DSP) steps like that followed for the recombinant PAI-2 product. The Gene-minus DSP material was fractionated on three different columns containing Q-Sepharose, Ph-Sepharose and Tentacle ion-exchange resins. Fractions-off three DSP columns were tested for presence of any PAI-2 by ELISA and immunoblot assays. Having ascertained the total absence of PAI-2 in any of Gene-minus DSP fractions from three columns, the fractionated materials were designated Q-Sepharose, Ph-Sepharose and Tentacle fractions, On the other hand, Gene-minus preparations were fractionated on chromatography columns with fresh resins and therefore used as controls. It was important to establish whether Gene-minus preparations should not contain any PAI-2 before using them for immunisation.

Immunisation of rabbits

The protein content of fractionated DSP preparations was determined by BioRad BCP assay. Altogether, 50 New Zealand white rabbits were divided randomly into five groups of 10 each. A dose of 500 µg of DSP fractionated protein was completely homogenised in 1:1 proportion with Freund's complete adjuvant and injected at 10 different sites SC after clipping the body- coat on the back of each rabbit as primary immunisation. Subsequently 500 µg of fractionated material were mixed with Freund's incomplete adjuvant and given as booster injection. Further injections were given 4 weeks apart and injected similarly into 10 different sites. Two assays, ELISA and Immunoblot assays were employed for validating the antibody response against Gene-minus preparations.

Antibody response in rabbits to gene-minus yeast antigens

Rabbits were immunized in two phases. Animals were given a second booster and sera were harvested and retested with homologous antigens. Data in Figure 2 show that the rabbits have responded strongly to all antigens following a second booster. Sera were positive even at 1/100,000 dilution. These sera after 2nd booster have been designated "hyper-immune". Those rabbits, which attained "hyper-immune" status, have been periodically bled and sera harvested.

Bleeding of rabbits and harvesting of Gene minus antisera: Generally, rabbits were bled by vene-puncturing the marginal veins. The bleeding dates followed, and sera were collected for baseline estimation (NRS), prior to 1^{o}booster, 2w after the booster and 2, 4 and 6 w after 3^{o}injection. Fourth injection was also given solely as a maintenance dose and sera were harvested by bleeding rabbit's 2w post 4 injections. Sera were collected and stored at -40^{o}C until use.

Immunization of Sheep

A group of 4 crossbred sheep were immunized each with 1 mg of 2^{o}Q-Sepharose emulsified in FCA subcutaneously and boosted 28 days later with 500 µg of Gene-minus preparations and animals were bled and sera harvested.

Levels of HCPs in the r-PAI-2 therapeutic product

A thorough investigation of HCP impurity was vital to establish the product purity. This again was based on the sensitivity of analytical methods followed for detecting HCPs. Figures 91 to 95 per cent homogeneity was mentioned as desirable for therapeutic proteins [6]. Figures such as 99 to 99.99 per cent were difficult to achieve [25]. Highly sensitive analytical techniques are essential to achieve that level of purity. There was no unequivocal figure indicated for host-contaminant proteins [7]. It has been shown

that a dose of 0.3 µg contaminant per gram of product produce significant physiological effects [3]. From that point of view, it was generally accepted that a microgram (µg) level of extraneous DNA was required to show any in vitro oncogenic effect.

PAI-2 product and the possible HCP

PAI-2 manufactured at Biotech Aust Pty Ltd, Australia using a eukaryotic system. The host *S. cerevisiae* with the integrated vector bearing the PAI-2 gene was propagated in large scale using defined media. The following mid-stream and down-stream process operations were applied to obtain PAI-2 in as high purity as possible. Several scientists were involved in this exercise. These multistep process operations had certainly minimised and reduced the HCP impurity. In brief, the fermented PAI-2 preparations were subjected to mid-stream processing (MSP) and filtered through 0.2 µm membrane. The filtered product was further subjected to several steps of down-stream (DSP) and chromatographically purified sequentially through 1^{o}Q-Sepharose, Phenyl Sepharose and 2^{o}Q-Sepharose. Then, a new ion-exchange Tentacle resin was introduced replacing 2^{o}Q-Sepharose to achieve further purity. In case of r-PAI-2, 5 ng of HCP in every 50-µg dose is acceptable. This works out to be about 0.01% or 100 ppm or 1 part of HCP in every 10,000 parts of PAI-2.

Apart from developing appropriate techniques to purify the product, simultaneously there should be parallel program for developing appropriate process specific analytical techniques to monitor the product purity at different level of manufacturing. In view of the level of sensitivity expected (picogram level/ppm) and due to many samples to be assayed during manufacturing, an ELISA system for monitoring the HCP during process operation as well as in the final product was developed [12].

Requirements for HCP-ELISA system

The condition for an HCP-ELISA varies as per the need and type of product. However, some information is available on the detection of host-cell protein contaminants in r-DNA based biopharmaceutical products such as human insulin [9], human growth hormone [10,4] produced in *E. coli* and human erythropoietin in mouse fibroblast cell line [11]. There is as yet no HCP-assay described for r-DNA products derived from *Saccharomyces* system. Moreover, no antibodies either to *S. cerevisiae* to its Gene-minus variants are commercially available. With this, we considered the importance if such an assay in validating the products. Nevertheless, we anticipate some difficulties and technical limitations during design and construction of such assays. The following are regarded as the basic need to develop an HCP-ELISA system:

(i) Gene-minus host (yeast) antigenic (null cell) preparation.
(ii) Polyclonal antibodies (PAb) raised in rabbits and sheep.
(iii) PAb to micro-components of 2^{o}Q-Sepharose by immunizing Rabbits with affinity isolated antigens.
(iv) Separation and purification of PAbs.
(v) Conjugation of PAbs with Horse Radish Peroxide enzymes.
(vi) Setting up ELISA- Assay optimization and standardization.
(vii) Data analysis.
(viii) Stability and QC reagents.
(ix) Validation of ELISA system.
(x) Preparation of SOPs, Documentation and Reporting.

It is hoped that this test system would provide appropriate guidelines and test systems for QC and QA purpose and to satisfy the Regulatory Authorities for approving PAI-2 as a therapeutic product.

Initial Enzyme-Linked Immunosorbent Assays

A variety of ELISAs have formulated to detect and to quantify HCPs as "impurity". A double antibody sandwich ELISA was initially employed to demonstrate any presence of PAI-2 in the Gene-minus preparations. Mouse anti-PAI-2 MAb (Biopool) was used as the capture and the Goat anti-PAI-2 PAb as the detector. Briefly, the microtitre plates (Maxisorp; Nunc) were coated with mouse anti-PAI-2 MAb (Biopool) (capture antibody @ 10 mg/ml concn) in the coating buffer supplemented with BSA (0.01%) for 16-18h (overnight=O/N) at room temperature (RT)), and subsequently blocked with 3% blotto for 45 min at 37 C. A panel

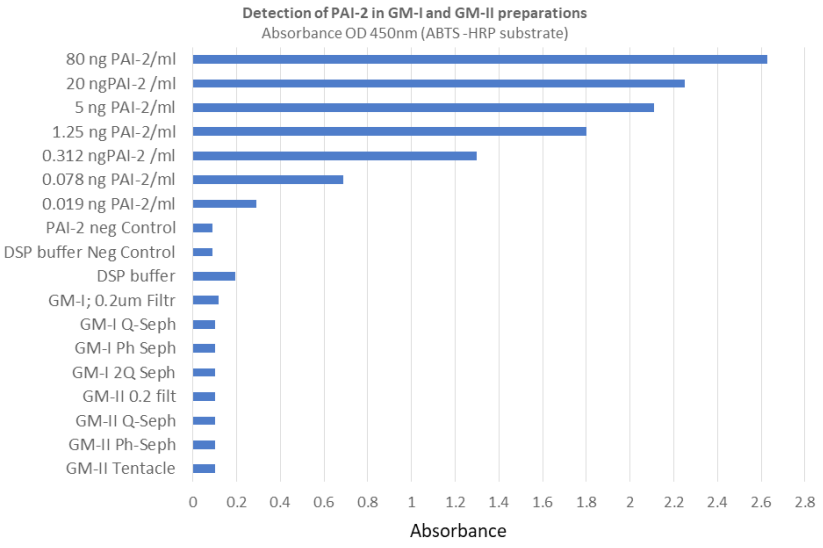


Figure 1: The ELISA assay is sensitive and reacts with as low as 0.019 ng/ml of PAI-2 (Actually, it detected as low as 0.0019 ng in 100 µl assay volume). All control reagents and buffer showed reactivity as low as 0.2 OD.

of PAI-2 standard was set up (a serial dilution starting 80, 20, 5, 1.25, 0.3125, 0.078 and 0.019 ng/ml) as a reference. Four each of concentrated and dialysed Gene-minus preparations (10 mg/well in 100 ml volume) along with appropriate DSP buffers as controls were reacted and incubated for 2 h at 37°C in ELISA plates. The plates were washed and further reacted with the detector antibody, Goat anti-PAI-2 PAb (Biotech) at 2.5 mg/ml concn. The conjugate, Donkey anti-sheep/goat Ig-HRP (Silenus) at a 1:1000 concn was used and developed with TMB substrate (Behring). The plates were read at 450 nm (Figure 1).

Gene-minus Yeast protein capture ELISA

A Gene-minus yeast HCP-ELISA requires a considerable number of reagents developed in-house. The expected final format of HCP assay was a 2-site sandwich ELISA [12]. Microtitre plates were coated with Gene-minus Rabbit PAb (10 µg/ml) and then reacted with standard Gene-minus antigen or with final r-PAI-2 product containing HCPs. In this assay the HCPs were captured by the protein A/G purified Gene minus rabbit antibody immobilised on to the solid phase and non-specific sites were blocked with 5%PVP+5%Mannose and the captured HCP were detected through HRP-labelled Gene-minus detector antibody (Rabbit or Sheep PAb). Required quantities of these antibodies were purified for labelling with HRP enzyme. In some cases, Behring standard was also used, and the test PAI-2 preparations was also tested. After incubations of 2h detector antibody for Gene -minus sheep PAb labelled wit hHRP or Behring Conjugate was used as the detector antibody and incubated for 2h at 37C. TMB was used as the substrate. The plates were read at 450 nm and relative quantity of HCP in DSP-III preparations was calculated by referring to the standard.

Data analyses and calculation of HCP content in PAI-2 preparations

Triplicate OD readings of standard and test samples were subtracted with the appropriate blank and resultant absorbance was subjected to 4-parameter Curve-fit program using Delta-Soft II 3. 3B and resultant data were analysed. The HCP content in PAI-2 preparations were determined by using a standard graph. The amount of HCP (in ng/ml concentration) present in the test sample (mg/ml) were tabulated and the ppm of HCP was determined by the following formula [11].

$$\text{ppm} = \frac{\text{ng/ml HCP x Dilution factor}}{\text{mg/ml protein (PAI-2)}}$$

Results

Determination of antibody titre in Rabbit Hyper-immune sera

Antibody level in hyper immune sera was tested in ELISA using appropriate homologous antigen. ELISA conditions [12] were as follows. Microtitre (Nunc Maxisorb Cat # 4-42404) plates were coated with Gene-minus antigen (25 µg/ml concentration) diluted in coating (carbonate/bicarbonate buffer) buffer supplemented with 0.01% BSA and left overnight at RT. After incubation, the plates were blocked with 3% blotto for 45 min at 37C. After a brief wash appropriately diluted serum samples from immunised rabbit /sheep were added and incubated at 37°C for 2 h in a humid chamber. After incubation the plates were washed and the second conjugate antibody (In case of rabbit sera, Sheep anti-rabbit Ig-HRP, in case of sheep, Donkey anti-sheep Ig-HRP conjugated to horse radish peroxidase (HRP) has been added as the detector antibody. Substrate consisted of ABTS and H_2O_2. Plates were read in a plate reader (Bio-Tek Instruments EL309 Microplate Auto Reader) using a dual wavelength (415/490 nm) and relative antibody titre were expressed in the form of histograms using Cricket Graph Program.

Figure 1 showed mean+/-SD relative absorbance (450 nm) of PAI-2 standards, GM-I and GM-II preparations along with PAI-2 negative control and DSP buffer controls. DSP buffer control showed an absorbance of OD=0.195. As low as 0.019 ng/ml PAI-2 standard showed an absorbance of OD=0.281 indicating that this assay has a sensitivity of 0.019 ng PAI-2 per ml of sample. None of Gene-minus preparations showed higher than the OD values of DSP medium control indicating the Gene-minus preparations contain no detectable level of PAI-2 (ie, <0.019 ng PAI-2/ml of Gene minus preparation).

Immunoblot Assay

Immunoblot assay was performed by electrophoresing the Gene minus preparations on SDS-PAGE gel and transferring the proteins onto the nitrocellulose membrane which were then reacted immunologically and then visualised with appropriate detector antibody. In brief, Gene-minus preparations were electrophoresed using 5 mg/well in Novex precast gels (12% Tris-Glycine gel 1.0 mm x 15 well Cat EC60055) along with PAI-2 standards (500 ng/well; +ve control) and Low MW (BioRad; Cat 74463, prestained low range MW) markers under reduced conditions in X-Cell II (Novex mini-cell) at 50 mA for 40 min in SDS-PAGE buffers. The gel was taken out of the cast and placed on a nitrocellulose (Protran BA 0.2 mm Cat 401396; Schleicher & Schuell) membrane which is pre-layered over 4 layers of filter papers (Whatman chromatography paper 3 mm Cat # 3030917) soaked with semi-dry electrophoresis buffer (SDS 0.786 g, Tris 11.62 g, Glycine 5.86 g, Methanol 400 ml, Distilled H_2O qs 2 litre, pH 9.2) on a semi-dry electrophoresis unit (Ancos Semi-dry electrophoresis Unit). The gel was arranged carefully avoiding stretching of individual lanes. Another 4 layers of pre-soaked filter paper were placed, and care was taken to avoid air bubbles and to keep the entire layers soaked with the buffer. The cathode plate was placed, and protein was transferred onto the membrane at 110 mA for 50 min. The membrane was washed in Tris-saline buffer 6x and blocked with blotto for 2 h on the rocker. Then a 100 ug of anti-PAI-2 MAb (MAI-21, *mouse origin* Biopool) was added to the blotto and incubated O/N at 4°C on the rocker. On the following day, the membrane was washed 6x with TS buffer and reacted with Sheep anti-mouse Ig-HRP conjugate 1:1000 in 20 ml of Tris-saline buffer for 1 h. Following the reaction, the membrane was thoroughly rinsed 8x with Tris- saline, substrate added and colour, an indicator for presence of any PAI-2, was developed for 30 min. Except the PAI-2 standards none of the Gene minus preparations showed any bands in the immunoblots (<0.019 ng/ml) of PAI-2 (Data not shown).

Separation of Ig by sequential precipitation with caprylic acid and ammonium sulphate

GM-PAbs present in QS- and TAE Grand-pool sera were purified by caprylic acid and ammonium sulphate precipitation. For a pilot scale 10 ml of Grand-pool QS –sera was subjected to sequential precipitation of Ig with caprylic acid and Ammonium sulphate. Immunoblot demonstrated reactivity of Ig. For a preparatory scale separation, 250 ml serum was subjected to precipitation.

Construction of QS-Ligand Column

Affigel-10 (BioRad) was used to immobilise the gene-minus ligands (QS fraction) on to the resin. 10 ml of Affigel-10 slurry was washed on a glass fritted funnel with chilled MilliQ H_2O and then with the coupling buffer (0.1M Hepes + 80mM $CaCl_2$ in MilliQ H_2O), and transferred into a bottle. Host cell protein preparation (Gene-minus II QS 55 mg total in 5 ml) was mixed and coupling was performed at neutral pH at 4C for 4 h. Then the remaining reactive sites were blocked with 1M ethanolamine for 1h. The coupled Affigel was packed into a column and then washed with the binding buffer (PBS 0.1M; pH 7.4) and eluate was checked at OD_{280}. The column was further washed with elution buffer (Glycine-HCl/Dioxan pH 4), PBS, 1M NaSCN and finally equilibrated with PBS. The pilot scale column bed volume was ~5 ml. For preparatory scale, a total of 168 mg of QS-ligand was used to couple 50 ml of Affigel-10.

Construction of TAE-ligand column

The procedure adopted was as above with QS-ligand column, but only 21.5 mg ligand for 10 ml Affigel was used. The TAE fraction was concentrated in an Amicon cell (YM-10) by a factor of 16 prior to coupling. The final bed volume was 5 ml.

Affinity Chromatography and analysis of fractions

Affigel -10 ligand columns were attached to LKB Bromma Chromatography System (2211 SuperRac; 2238UVI Cord S-II; 2132 Microperpex perisaltic pump; 2210 chart recorder) and batches of QS-Ig/TAE-Ig were loaded on to the column and allowed to bind to the ligand in a continuous flow providing a closed circuit for a minimum of 1h. Then the column was washed in PBS 0.1M pH 7.4) and eluted in Glycine-HCl (pH 4.0). Unbound and affinity purified fractions were identified by monitoring the UV absorbance of the column effluent (280 nm). Appropriate fractions were pooled and concentrated by YM-10 (Amicon) wherever necessary and Ig content was determined. The affinity purified and unbound pooled fractions were stored at -40°C until use.

Calculations: Protein (Ig) content of column eluate was initially checked at OD_{280} and the Ig content was expressed with the formula: OD_{280}x0.7=mg of Ig per ml x dilution factor x fraction volume= Total Ig content.

Assessment of affinity purified Gene-minus antibodies

The pooled fractions of flow-through preparations, affinity purified GM antibodies were assessed by immunoblotting against appropriate Gene-minus antigens.

Bradford Protein assay

Bradford Protein Assay (BioRad) was performed to overcome any interference at OD_{280} reading estimated pooled antibody preparations from TAE affinity purification for Ig content. A standard curve was set up each time with BSA ranging from 1-14 µg/ml. Reaction volume was 1 ml; the sample (e.g. 10 µl) is appropriately diluted with MilliQ H_2O (790 µl) and mixed with 200 µl Bradford Dye reagent and incubated for 15 min and read at 595 nm. The protein value was determined by following the Bradford Program on Spectrophotometer (Shimadzu UV 160A).

ELISA Data

The $OD_{415/490\,nm}$ readings were analysed using Delta Soft II 3.3B and relative antibody titre was expressed in the form of histogram/line graph using Cricket Graph Program. Antisera with an absorbance of >0.100 at 450nm was regarded as Positive and OD <0.100 was negative (Figure 2).

***Antibody titre in sheep*:** Four sheep (S-49, S-50, S-51, and S-52) were immunised with GM *Saccharomyces* antigens. Four sheep were bled following the first booster (Day- 45) and the resultant sera were tested in ELISA using 2° Q-Sepharose fraction of Gene minus yeast antigens. All individual sheep responded very vigorously with an antibody titre of 1/40,000. Purification of Ig was performed with similar protocol adopted for rabbit sera.

Immunoblot assay on Gene minus antisera

Gene-minus antigen preparations were electrophoresed along with MW markers in precast Novex minigels (1.5 mmx 10 well SDS-PAGE gels- 12% acrylamide, 2.6% bisacrylamide in Tris-glycine buffer: Cat EC 6008 using reduced sample with a concentration of 3 ug/well. Electrophoresis was performed in X-Cell II (Novex Mini-Cell) at 50 mA for 40 min. The gel was transferred on to a nitrocellulose membrane. After transferring the proteins on to the membrane, the membrane was taken out into a large Petri dish and washed 6x with Dist H_2O. Immunoblotting was performed in two ways.

(i) Standard Western blotting with 4-Chloronapthol as the substrate. In this method, the membrane was sliced into 10 strips according to individual well area on the gel. The membrane strips were blocked with 4.5 ml blotto and then reacted with 15 ul of GM antisera (1/300 dilution) and incubated O/N on the rocker at 4° C. After reacting with the target Rabbit sera, the individual strips were taken out into large petri-dish and washed 6x in Tris-saline and ultimately reacted with Sheep anti-rabbit Ig-HRP conjugate at 1/1000 dilution for 1h on a rocker. Following incubation, the strips were washed with Tris-saline 10x and then reacted with 4-Chloronapthol-HRP substrate and developed for 30 min and reaction was terminated by rinsing the strips with Dist H_2O. The strips were photographed, and upon drying, they were laminated and analysed for the reactive components in Gene-minus antigen preparations.

(ii) Immunogold silver staining (IGSS). Required number of plastic wedge-trays were arranged and a volume of 4.5 ml of blocking solution (Probe buffer Proprietary preparation, Behring was added to each tray. Individual strips were marked with a code for further

identification and briefly incubated in the probe buffer for 30 min and then 15 µl of GM antisera (1/300 dilution) was added. Trays were placed on a rocker in the cold room and incubated O/N for 16-18 h. Then strips were taken out and pooled together in a large Petri dish and washed 6x with Behring wash solution (Stock solution 10x strength: 6.05g Tris(hydroxymethyl-aminomethane), 10.5g citric acid, 8.36g sodium hydroxide, 100 g sucrose dissolved in 1 litre MilliQ H_2O (Dilute 1/10 for work solution.) on the rocker at RT. Then the strips were incubated in Auroprobe BL plus solution (Gold labelled anti-rabbit Ig) on rocker for 45 min at RT. Then reactants were discarded, and strips were thoroughly washed 8x in Behring wash solution and then 3x with Dist H_2O. Ultimately, the strips were exposed to the silver enhancing substrate solution (IntenSE; equal proportion of enhancer and initiator. The gold catalysed the reduction of silver ions (silver lactate) to metallic silver in presence of a reducing reagent (hydroquinone). The silver forms a dense black deposit around each gold particle. The signals are visualised by the gold induced precipitation of metallic silver resulting with a high contrast dark brown to black signal. Self -nucleation ie, random silver precipitation was prevented by removal of the substrate and rinsing with the Dist H_2O with 20 min. The signal was further developed and enhanced by repeating the above step once or twice if needed to further enhancement of signal. The strips which were developed with IGSS were finally washed in water, air dried and laminated.

***Separation of Ig*:** The reactivity of GM-PAbs against QS-ligand was demonstrated by 2D Immunoblot shown in Figure 4. The antisera reacted with several components with pI 4 -7 in the QS ligand. Some high MW components resolved in the region pI <4. There was no significant difference in the reactivity of antibodies in the Ig preparation compared to that demonstrated in the Grand-pool sera. A total of 75 mg immunoglobulin (9.4 mg/ Ig for each ml of Grand-pool sera) was purified from 10 ml of QS-Grand-pool sera and stored at -40°C.

Preparatory Scale of affinity purification of GM-QS-PAbs:

The column bed volume was 30 ml. About 400 mg GM-QS-PAbs in 28 ml (14.5 mg/ml) was bound by continuous flow in a closed-circuit system for 16h. A total of 200 fractions were collected and combined as follows: Unbound (120 ml; 375 mg); Affinity purified prep Peak- 1 (70 ml 35.23 mg), Peak- 2 (300 ml; 82.95 mg) Total affinity purified GM-QS-PAb=118.2 mg. Percentage yield of purified GM-QS-PAb =29.5% Protein estimation was done by Bradford protein Assay. All preparation were analysed by loading 100 µg each lane (Figure 3) where affinity purified QS-PAb peak-1 and -2 assayed on yield basis (Lane 5 and 6) flow through on volume basis (Lane 4) compared to input Ig preparation (Lane 3) and Ig in Grand Pool sera (Lane 2).

Caprylic acid and Ammonium sulphate precipitation yielded respectively 9.4 and 8.2 mg of Ig per each ml of QS- and TAE-antisera respectively. The immunoreactivity profile as visualised by 2D-immunoblot analyses (Figure 4) also showed high specificity to HCP components. Currently we have 995 ml TAE grand – pool sera that may yield about 8200 mg immunoglobulins. These preparations will be useful in setting up sensitive HCP assays for monitoring the purity of PAI-2 product.

Characterization of antibody response

It is of interest to observe the pattern of antibody response in different rabbits. Gene-minus rabbit antibodies are required for monitoring the HCP during process operations. The HCPs in the final product may represent those antigens identical to that of 2°Q-Sepharose fractions. In view of these, it is relevant to test the sera of first three groups with 2°Q-Sepharose antigens (Figure 5). Immunoblot analyses of a panel of rabbit sera (QS-and T- group) ph-S group following reaction with Tentacle fraction (Figure 5). Tentacle ion –exchange fractions were electrophoresed by SDS-PAGE and transblotted on to the nitrocellulose membrane. Ten strips were prepared and reacted with pooled sera and the strips were developed by IGSS. Immunoblots are shown in Figure 6. It also reflects on the DSP of PAI-2 preparation where type of HCP may vary at each stage.

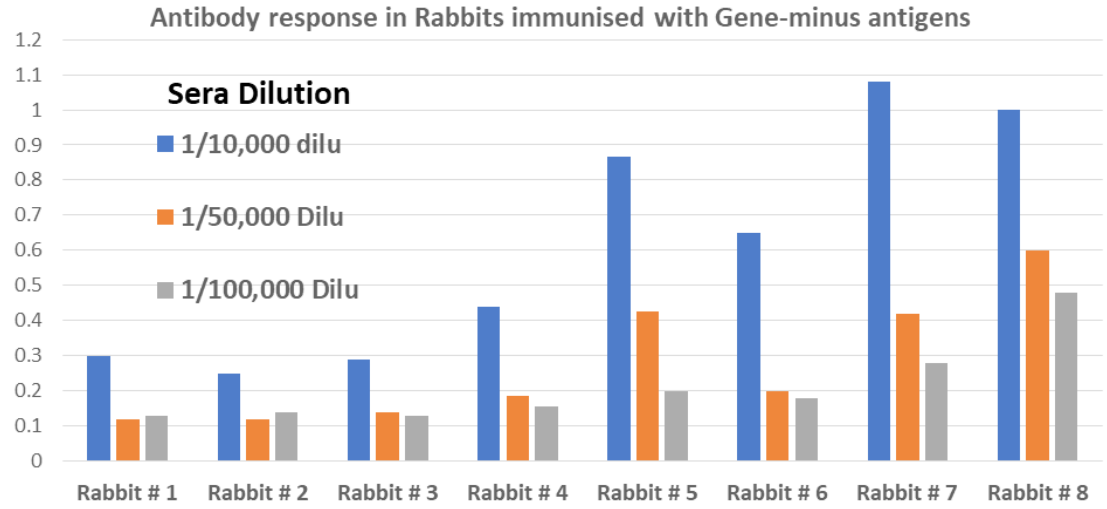


Figure 2: Antibody response of few selected rabbits for Gene-minus antigens. Rabbits have reacted with 1/100,000 dilution of sera samples.

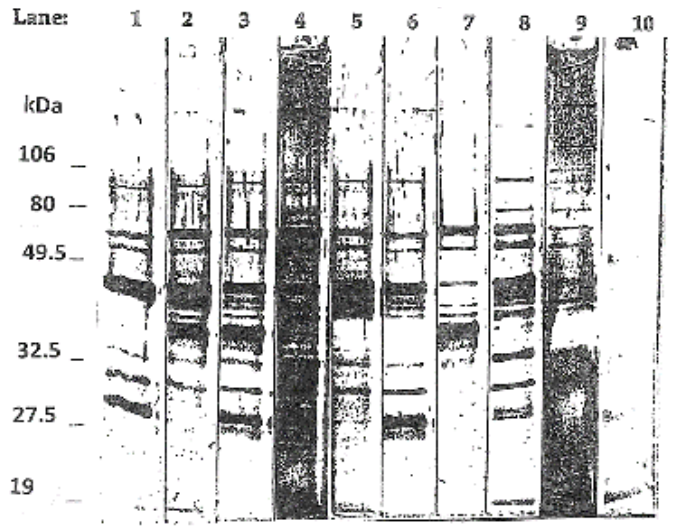
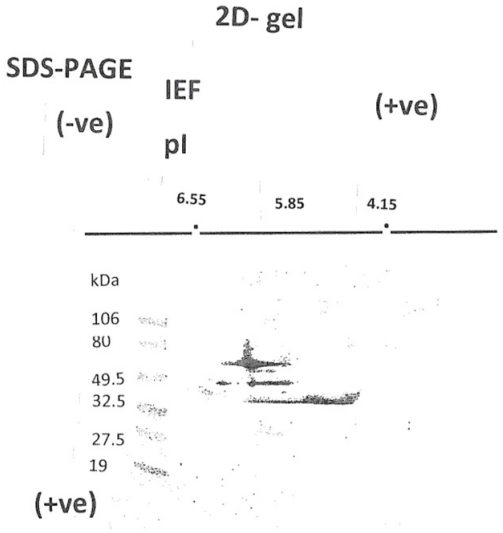


Figure 3: Immunoblot analyses of a panel of rabbit sera (QS- and T- group; 6w post 3 injection, Ph-S group 2 w post 3 injection and following reaction with Tentacle fraction. Tentacle ion - exchange were electrophoresed by SDS-PAGE and transblotted on to nitrocellulose membrane. Ten strips were prepared and reacted with pooled sera and the strips were developed by IGSS. Lane 1: mixed sera from two groups of rabbits in Ph-Separose+Tentacle groups, Lane 2: Mixed sera from two groups of rabbits in Q-Sepharose+Tentacle groups, Lane 3: Mixed sera from two groups of rabbits in Q-Sepharose+Ph-Sepharose. Lane 4 and 9: Tentacle ion-exchange fraction with AuroDye-Forte. Lane 5: Pooled sera from rabbits (R41-50; 6w post 3 injections in Tentacle group. Lane 6: Pooled sera from rabbits R31-40 2w 3 injections immunised with Ph-Sepharose. Lane 7: Pooled sera from rabbits R21-30, 6w post 3 injection immunised with Q-Sepharose. Lane 8: Behring affinity purified anti-GM1 antibody raised against 2 Q – Sepharose fraction of GM-1) 225 µg/strip. Lane 10: MW range.



IEF of Gene-Minus Fraction and 2D SDS-PAGE analyses followed by Immunoblot with Pooled Rabbit Sera

Figure 4: Gene-minus QS fraction was electrophoresed in 10 well SDS-PAGE gel and one strip was further 2D electrophoresed. IEF of QS fraction of 2D PAGE analyses followed by immunoblot with pooled sera. The antisera reacted with several components with pI 4-7 in the QS ligand. Some high components are resolved in the region of pI <4. High specificity to HCP molecules were observed.

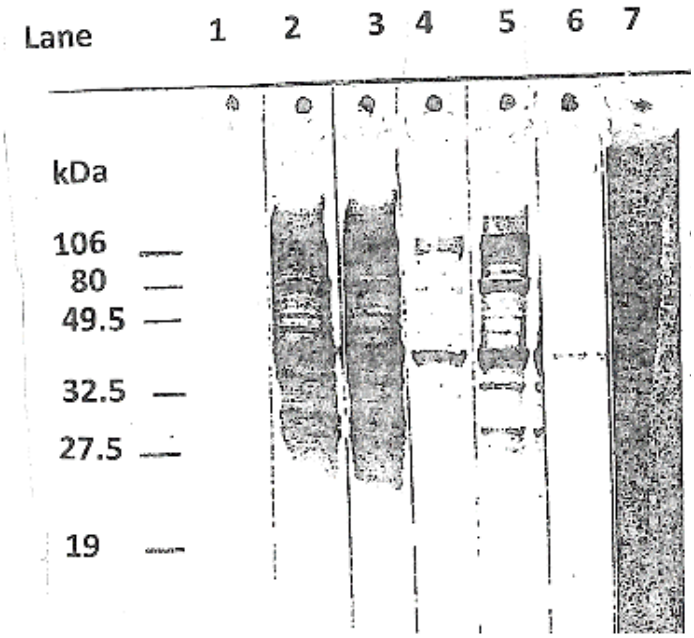


Figure 5: The column bed volume was 30 ml. About 400 mg GM-QS-PAb in 28 ml (14.5 mg/ml) was bound by continuous flow in a closed-circuit system for 16h. A total of 200 fractions were collected and combined as follows; All preparations were analysed by loading 100 µg into each lane. Legend: Lane-1 MW markers; Lane-2 Affinity purified Ig; Lane -3 Input Ig preparation; Lane-4 Flow through (volume basis); Lane- 5, Affinity purified preparatory Peak 2 (300 ml;89.25 mg); Lane- 6, Peak-1 (70 ml; 32.23 mg). Unbound 120 ml; 357 mg; Total Affinity purified GM-QS-Ig 118.2 mg. Percentage yield of affinity purified QS-Ig =29.5%. Protein estimation was done by Bradford protein assay where affinity purified QS-PAb Peak 1 and 2 assayed on yield basis. Flow through on volume basis (Lane 4) compared to input Ig preparation (Lane 3) and Ig in Grand-pool sera (Lane 2).

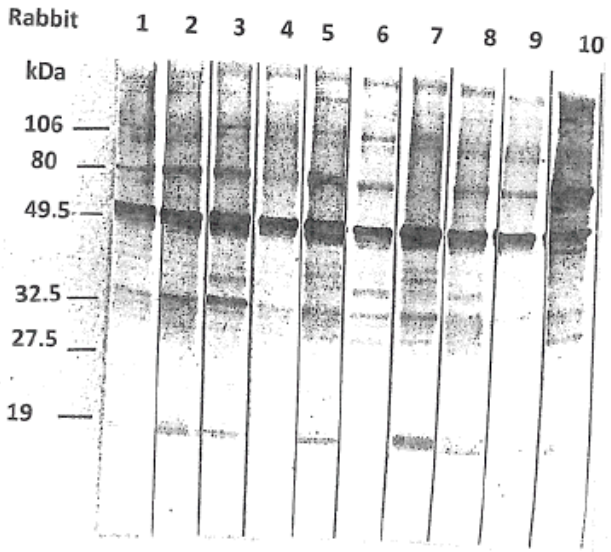


Figure 6: Rabbits immunized with Tentacle fractions (R-1 to R-10) and their sera were immunoblotted. Immunoreactivity was uniform and showed very high affinity antibodies to 50, 80 and >106 kDa components.

HCP-ELISAs

The main reason for choosing ELISA for monitoring HCP is due to the feasibility for automation, consistency in its performance, and due to least hazardous nature of the assay. Moreover, it is more attractive due to considerable amount of technical improvement such as 96- well format the ELISA plates, availability of a variety of defined enzyme labelled conjugates and ready-to- use substrates, automated plate reader, and software for evaluating the reagents [13]. Due to this flexibility ELISA system has been applied into several applications over the years. Based on this determination a mixture of 5%PVP +5%Mannose blocking of microtitre wells gave highest correlation co-efficient, which has been routinely adopted for HCP determination.

Three different types of conjugates were used.
(i) Rabbit antibody raised against yeast antigen and labelled with HRP (Behring Laboratories Marburg Cat # 3702). This is not a Gene-minus antibody conjugate.
(ii) Gene-minus Hyper immune serum found to be positive at 1/100,000 dilution was affinity purified and labelled with HRP enzyme (Gene-minus rabbit IgG-HRP conjugate)
(iii) Gene minus Sheep PAB was affinity purified and labelled with HRP enzyme (Sheep IgG-HRP conjugate). This conjugate would serve as complimentary to the second conjugate.

Indirect HCP-ELISA

DSP-III PAI-2 preparations containing yeast HCP were coated at 20, 10, 5 and 0.5 ug /ml concentrations on to microtitre wells. All wells in the plate were blocked with a blocking solution (5% PVP+5% mannose). Wells were blocked to nullify any non-specific epitopes. Gene-minus Rabbit PAb was used the detector antibody to specifically bind to HCP present, which were visualised with Sheep anti-rabbit Ig-HRP conjugate. Results are shown in Table 1.

Table 1: *HCPs detected by Indirect ELISA.*

DSP III PAI-2 Preparation	Protein comp (mg/ml)	HCP detected (µg/ml)	%	PPM
P124				
F3	16.7	1.68	0.01	100
F4/5	4.8	0.719	0.014	140
F6	12.9	0.08	0.0006	62

Standard (2°Q-Sepharose did not titrate well with rabbit antibody and the same reading was obtained at 20 µg/ml down to 5 µg/ml concentrations. Results obtained were not suitable for analysis in 4-Parameter Curve-Fit program.

Sandwich HCP-ELISA

Sandwich ELISA assays were more sensitive that other assays and known to be highly suitable for HCP determinations [4]. Different reagents were used to run the sandwich ELISA.

Assay conditions: Wells were coated with Behring coating antibody, Wells were blocked. Standard and controls were used. Detector antibody Behring Rabbit antibody labelled with HRP. TMB used as Chromogen. Results are shown in Table 2.

Table 2: *Sandwich HCP-ELISA.*

DSP III PAI-2 Preparation	Protein comp (mg/ml)	HCP detected (µg/ml)	%	PPM
P124-F3	16.7	218	0.109	1090
P124- F4/5	4.8	235	0.112	1120
P124-F6	12.9	142	0.07	700
P126-F3	15.8	246	0.113	1130

An aliquot of 200 µg/ml DSP-III PAI-2 preparation was found to be satisfactory for detection of HCPs. P124-F6 was found to be purer than other fractions tested in HCP ELISA.

Table 3: *Comparative ppm level of HCPs in DSP-III PAI-2 preparations.*

DSP III PAI-2 Preparation	Protein comp (mg/ml)	ppm HCP detected at	
		Roseville	Marburg
P124-F3	16.7	1090	3670
P124-F4/5	4.8	1120	2560
P124-F6	12.9	700	430
P126-F3	15.8	1 130	555

Marburg Laboratories detected higher amount of HCPs. It may be due to usage of their rabbit antibody –conjugate (non-Gene-minus) raised with yeast antigens. At Roseville, we carefully employed Gene-minus Rabbit antibody for coating and Gene-minus sheep antibody IgG-HRP as conjugate.

Comparison of HCP-ELISA data with the analysis performed at Behring Laboratories, Marburg. The DSP-III preparations were mutually compared with the HCP-ELISA.

Further resolution of HCP in DSP-III PAI-2 preparations

An attempt was made to further resolve the HCPs through Sephacryl 200 column (Figure 9). P124-F6 preparation was subjected to further purification through S-200 (Sephacryl) column and 7 fractions were eluted off and then tested for the presence of HCP.

Assay Conditions

Coating Antibody: Microtitre wells were coated with protein A/G purified rabbit (10 µg/ml concentrations).
Blocking: Blocked with 5%PVP+5%Mannose
Test sample and standard
Reacted with standard Gene minus 2°Q-Sepharose fraction (800 to 12.5 ng/ml and other test fractions (Fraction #3 to 9).
Second Antibody: Gene minus sheep sera diluted 1/500 followed by Donkey anti-sheep Ig HRP at 1/1000 dilution.
Substrate TMB

Table 4: *HCP values determined by HCP-ELISA.*

DSP III PAI-2 Preparation	Protein comp (mg/ml)	HCP detected (µg/ml)	PPM
P134-F5	22.6	638	2823
P134-F6	5.8	620	3050
P134-F7	34.4	318	1540
P153-P2	4.8	1105	227
P155-F4	5.2	850	162
P157-F5	2.8	970	336

The last three fractions showing the presence of HCPs were analysed by Immunoblotting, which has been presented in Figure 8.

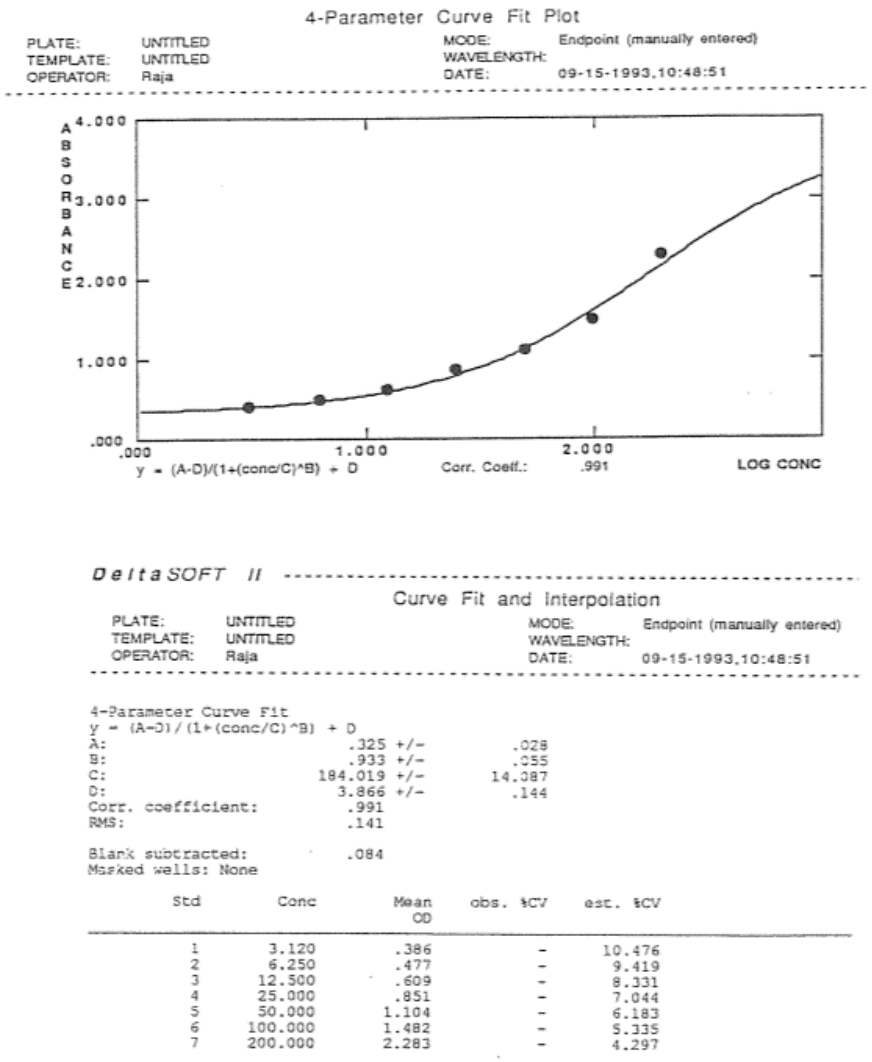


Figure 7: 4-Parameter Curve Fit and interpolating Delta Soft II programme showing coefficient variation to standard HCP detection curve ranging from 0 to 3.12.

Figure 8: Scanning of HCP (PAI-2) control (A) and P153 (Peak 2) 227 ppm (B), P155-F4 162 ppm (C) and P157 -F5, 336 ppm (D) (refer to Table 4) of HCP immunoblotted. HCP level recorded from 336 to 162 ppm.

Data analysis 4-Parameter Curve Fit

The plates were read at 450nm was quantified using Gene-minus 2o Q-Sepharose as the HCP standard. Results are in Table 3 and Figure 7. The results that yielded at two different laboratories (Marburg vs Roseville) are compared. Table 4 further delineated the HCP content in different fractions of DSPIII and some last three fractions were compared by immunoblotting (Figure 8).

Data in Figure 9 show that sizing resin (S-200) has effectively shifted HCP and the first two fractions (F-3, F-4) contained nearly 84% of HCP (Out of 2217 ng HCP resolved fractions 3, 4, 5 contained 1869 ng HCP. The bulk of PAI-2 was in fractions 4 to 8 (14,570 ng, which contained 226 ppm HCP). It works out to be 1.55% HCP that is minimal. These analyses showed that purification of r-PAI-2 through sizing resin (S-200) resulted with minimal (1.5% of HCP) level of HCP in the final product.

Discussion

The work reported here has laid the foundation for determination of HCPs in rDNA bio-pharmaceutical products. Identification and quantification of protein contaminants in the rDNA based therapeutic products have always been a problem [14]. Therefore, there is a strong emphasis on the definitive determination of HCPs in r-DNA-based bio-pharmaceutical products. The detection of host cell proteins requires a highly sensitive assay to detect at ppm levels [15]. The potential biological and immunological consequences of HCPs made it mandatory to go for highly sensitive assays. Immunoassay has been considered as the basic "assay of choice" and sandwich or two site immunocapture ELISA tests have been emphasized as essential features in the development of r-DNA therapeutic protein products [4,9-11]. Generation of very high titre antibodies to Gene-minus (null cells) yeast proteins is a major achievement. The fact that some of the manipulations performed during immunization

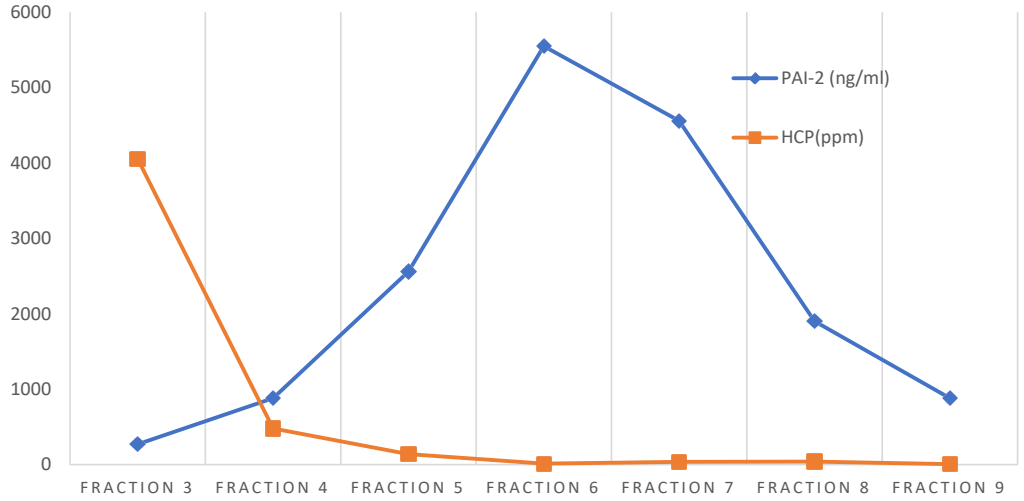


Figure 9: Sizing resin (S-200) has effectively shifted HCP and the first two fractions (F3, F4) contained nearly 84% of HCP. The bulk of PAI-2 were in Fraction 4,5,6,7, and 8 (14570 ng) which contained 226 ppm of HCP (works out to be 1.55% of HCP).

schedule had enabled us to achieve this target. We generated Gene-minus antibodies in two species and have been affinity purified and labelled with appropriate enzymes and used in capture-ELISAs. We have constructed HCP-ELISA which is sensitive enough to detect 200-250 ppm of HCP. Typical impurities in protein pharmaceuticals are endotoxins, host-cell and media proteins, monoclonal antibodies, and defined production proteins used in the purification steps, DNA, infectious agents and a variety of product variants due to deamidation, oxidation, and aggregation and due to proteolysis [2]. What are the possible impurities one would expect with r-PAI-2 preparations? Host contaminants may be varied types. Yeast derived cellular (null cells) materials [16] obviously may form the bulk of HCPs. A wide range of cellular envelop cytoplasmic components can be expected. In addition, glucans, otherwise known as yeast cellulose, an insoluble polysaccharide; highly polymerised mannan, ie yeast gum and chitinous cell wall components. A variety of enzymes such as invertase, melibiose, acid phosphatase, catalase, and amylase are expected. *Saccharomyces* yeast contains 100- fold excess of invertase with 270 kDa. Amongst proteins, cell wall contains 12-17 amino acids such as glutamic (18%), aspartic (13%), leucine, alanine, serine, threonine etc.

The fermentation media contain relatively low molecular weight materials (Ammonium sulphate- 19g/l; glucose-50g/l, KH_2PO_4-3.1g/l $MgSO_4$ -10g/l, 0.2M $CaCl_2$-10 ml/l, trace elements 0.4 ml/l, Vitamin solution 10 ml/l, citric acid 10.5 g/l, yeast extract 900g/25 l; L-histidine HCl-64g /25 l. Some of the ingredients of fermentation media may be retained in the DSP-III preparation.

The buffers used the column chromatography consist of 20 mM glycine, 5mM EDTA, 10 mM β-Mercaptoethanol, 50 mM K_2HPO_4 and 180 mM NaCl.

As we are not using affinity columns, there is no of contamination with monoclonal antibodies and the product being intended for external use, elimination of infectious agents is of conjecture. Most of the media components and analytical chromatographic materials are defined ones and can be traced down. Host-derived polypeptides are expected to retain even in DSP-III preparation. However, their presence can be tracked down by using immunoassays employing highly avid anti-yeast antibodies.

As per the experience of Genentech scientists approximately 750 separate tests have been performed during manufacturing and release process of human growth hormone product [3-5]. Immunoassay (Sandwich HCP-ELISA) was found to provide specific and sensitive detection of HCPs [12]. They also found some non-immunogenic contaminants for which no antibodies were formed [8]. With our approach of cascade immunization, we successfully generated antibodies even to the micro-components of HCPs. A combination of complimentary analytical methods and process validation would provide the best assurance for minimal contaminants [17]. Recently HCP testing by ELISAs have reviewed by Genentech scientists [18] and have reviewed in terms of use of Chinese Hamster Ovary (CHO) cells for propagation of a variety of therapeutic proteins. As commonly accepted by the Biotech industry as well as by health authorities, the limits of HCP in biologics have been 1-100 ppm (or 1-100ng of HCP per mg of therapeutic protein reviewed by Eaton [19] and also by Champion et al. [20] and this level was essentially driven by the detection sensitivity [4] and the range of the analytical methods at the time. There are new approaches that are applicable for understanding residual HCP that can facilitate assessments of safety risk and potentially lead to better control of process impurities in the therapeutic products. Recent developments included liquid chromatography mass spectrometry (LC-MS) which are current state- of- the art tools for HCP measurement [23,25].

Acknowledgements

This work is performed at Protein Chemistry Department Biotech Aust Pty Ltd 28, Barcoo St Roseville NSW 2069. Several Scientists were involved in manufacture of recombinant PAI-2 products. Special thanks are extended to Neil Goss who inducted me to the task of characterization and validating HCPs, Thanks to Andreas Meister (Protein Chemistry) and other scientists, their help and co-operation were greatly appreciated to Fermentation (Ken Bertram), Chromatography (Pat Pilling), and animal experimentation (Les Horvath). Gene-minus S cerevisiae 50 litre production was supplied to initiate and to develop methods and document procedures for Host-Cell Contaminant Assay.

References

1. Botstein D. Why Yeast?. Hospital Practice. 1991; 26: 157-161.
2. Botstein D, Fink GR. Yeast: An Experimental Organism for 21St Century Biology. Genetics. 2011; 189: 695-704.
3. Anicetti V, Hancock WS. Analytical considerations in the development of protein purification processes. Bioprocess Technol. 1994; 11-36.
4. Jones AJS. Sensitive detection and quantitation of protein contaminants in rDNA products. The Impact of Chemistry on Biotechnology. 1988; 193-201.
5. Anicetti VR, Keyt BA, Hancock WS. Purity analysis of protein pharmaceuticals produced by recombinant DNA technology. 1989; 7: 342-349.
6. Wang X, Hunter AK, Mozier NM. Host cell proteins in Biologics development: Identification, Quantitation and Risk assessment. Biotechnology Bioengineering. 2009; 103: 446-458.
7. Garg VK, Costello MA, Czuba BA. Purification and Production of therapeutic grade proteins. Bioprocess Technology. 1991; 12: 29-54.
8. Tam JP. Physiological effects of transforming growth factor in the new born mouse. Science. 1985; 229: 673-675.
9. Baker R, Schmidtke JR, Ross JW, et al. Preliminary studies on the immunogenicity and amount of Escherichia coli polypeptides in bio-synthetic human insulin produced by recombinant DNA technology. Lancet. 1981; 2: 1139-1142.
10. Anicetti VR, Fehskens EF, Reed BR, et al. Immunoassay for the detection of *E. coli* proteins in recombinant DNA derived human growth hormone. J Immunol Methods. 1986; 91: 213-224.
11. Pauly JU, Siebold B, Schulz R, et al. Development of an ELISA for the detection and determination of contaminating proteins in recombinant DNA derived human erythropoietin. Behring Institute Mitt. 1990; 86: 192-207.
12. Voller A, Bidwell D. Enzyme-Linked immunosorbent assay. Manual of Clinical Immunology. 1986; 99-109.
13. Ishikawa E. Hashida S, Kohno T, et al. Ultrasensitive enzyme immunoassay. Clin Chim Acta. 1990; 194: 51-72.
14. Landis JB. Analytical challenges in biotechnology. The Impact of Chemistry on Biotechnology. 1988; 174-176.
15. Gueriguian JL, Chiu YYH. Clinical validation of recombinant products. Biotechnologically derived Medical Agents. 1988; 122-135.
16. Phaff HJ. Structure and biosynthesis of yeast cell envelope. The Yeasts. 1971; 135-210.
17. Goey CH, Alhuthali S, Kontoravdi C. Host cell protein removal from biopharmaceutical preparations: towards the implementation of quality by design. Biotechnology Advances. 2018; 36: 1223-1237.
18. Zhu-Shimoni J, Yu C, Nishihara J, et al. Host cell Protein testing by ELISAs and the use of Orthogonal Methods. Biotechnology Bioengineering. 2014; 111: 2367-2379.
19. Eaton LC. Host cell contaminant protein assay development for recombinant biopharmaceuticals. J Chromatography A. 1995; 705: 105-114.
20. Champion K, Madden H, Dougherty J, et al. Defining your product profile and maintaining control over it part 2. Bioprocess Int. 2005; 52-57.
21. Mattanovich D, Branduardi P, Dato L et al. Recombinant protein production in Yeasts. In Recombinant Gene Expression Review & Protocols Third Edition Springer Science 2012, 329-357.
22. Gomes AMV, Carmo TS, Carvalho LS et al. Comparison of yeasts as hosts for recombinant protein Production. Microorganisms 6, 2018, 38, 6020038.
23. Pilely K, Johansen MR Lund KR et al. Monitoring process-related impurities in Biologics- host cell protein analysis. Analytical and Bioanalytical Chemistry 414 (2022) 747-758.

24. Kim H, Yoo SJ & Kang HA Yeast synthetic Biology for the production of recombinant therapeutic proteins FEMS Yeast Research. 2015; 15: 1-16.

25. Geisow MJ Characterizing recombinant proteins. Biotechnology. 1991; 9: 921-924.

Matter of Interest to HCPs in Recent AstraZeneca COVID-19 Vaccine.

Researchers in Germany report substantial amounts of human and viral proteins present in AstraZeneca's coronavirus vaccine. AstraZeneca's vaccine contains a chimpanzee adenovirus genetically engineered to avoid its replication and instead Covid spike protein in people receiving the vaccination. The human protein impurities- mostly heat shock and cell scaffold proteins come from the human kidney cell line used to generate the chimp adenovirus. One vaccine lot contained 12.5 µg of virus in one dose and about 22 µg of non-virus proteins. Mass spectrometry of three vaccine lots shows between 45 and 71% of the vaccine consisting of viral and Mammalian proteins, with the rest being the active adenovirus vector ingredient ChAdOx1 nCOV-19. Contaminants were almost entirely absent in all three lots (Lea Krutzke et al. 2021 Research Square DOI 10.21203/rs.3rs-477964/v). Immunologist Hildegund Ertl at the Wistar Institute in Philadelphia, USA says that FDA will allow such proteins to be present, usually in the nanogram per dose range. But the AstraZeneca vaccine is in the microgram range, so pretty high, she says. Johnson&Johnson adenovirus vaccine is linked to fewer thrombotic side effects while these seem to be absent in the other adenovirus vaccine, Sputnik V.