BIOLOGIJA. 2006. Nr. 3. P. 4–7

Expression and characterisation of rubella virus capsid protein in yeast cells

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In the present study, we expressed rubella virus (RV) capsid protein (C) in yeast *S. cerevisiae* cells. Two different methods for purification of recombinant C protein were employed: CsCl gradient ultracentrifugation and nickel chelation chromatography. The yield of recombinant C protein was approximately 3.2 mg of purified protein from 1g of wet yeast biomass. The antigenic characteristics of recombinant C protein purified in different ways were further evaluated by indirect IgG ELISA with RV-positive and RV-negative human serum specimens. Recombinant C protein purified using CsCl gradient ultracentrifugation possessed a higher antigenicity as compared to that purified by nickel chelate chromatography. The results indicate that the recombinant C protein has a potential for use in detection of human IgG antibodies against RV. The yeast-expressed rubella C protein is a promising antigen for the development of diagnostic tools in serology.

Key words: rubella virus, recombinant capsid protein, yeast, indirect IgG ELISA

INTRODUCTION

Rubella virus (RV) is a human pathogen which causes rubella, or German measles. RV is a sole member of the genus *Rubivirus* within the family *Togaviridae*. The virus is transmitted from person to person via respiratory aerosols [1]. Rubella is normally a mild, self-limited disease, but it may cause foetal damage during the first trimester of pregnancy. The virus can cross the placenta, replicate in the foetus and induce birth defects known collectively as congenital rubella syndrome, most commonly involving heart defects, cataracts, deafness and mental retardation [2]. Thus, serological tests to determine the immune status in women of childbearing age and tests to diagnose RV infections in pregnant women are of great importance [3].

The RV virion consists of a genomic, single-stranded RNA enclosed in a quasispherical capsid composed of multiple copies of the viral capsid protein, which is in turn surrounded by a lipid bilayered envelope in which two virus glycoproteins, E1 and E2, are embedded [4].

It is known that human rubella virus infection induces specific immunoglobulin G (IgG), IgM and IgA antibodies against structural virus proteins which all have pathogenetic significance and diagnostic value [5]. RV-specific immunity in humans is usually assessed by measuring RV-specific IgG and IgM antibodies in serum. Individual specific antigenic RV structural proteins are required for the serological diagnosis of rubella infections [6, 7].

In general, the diagnostics of viral infections is based on serological methods. As an alternative to serological tests based on native virus antigens, highly specific serological tests could be developed on the basis of heterologously expressed viral structural proteins.

The aim of our study was to express the RV capsid protein and to study the suitability of the recombinant antigen as a diagnostic reagent in RV-specific antibody assays.

MATERIALS AND METHODS

Chemical synthesis of rubella virus C gene optimized for expression in yeast

Rubella virus RA27/3 strain capsid gene (C) (Genbank accession No. X14871) was chemically synthesized in order to optimize codons for expression in yeast *Saccharomyces cerevisiae*. Codon optimization and gene synthesis were performed by the GenScript Company (Piscataway, NJ, USA). The C gene sequence was provided in plasmid pUC18.

Generation of yeast expression plasmids

The capsid gene was cloned into the yeast expression vector pFX7 which contains galactose inducible GAL10-PYK1 hybrid promoter. For the generation of 6His-C-fused recombinant protein pFX7, derivative pFX7-6His

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was used. Insertion into this vector allows generation of protein harbouring a His tag at the amino termini [8]. The resulting plasmids pFX7-RC and pFX7-6His-RC were transformed into *S. cerevisiae* AH22 derivative 214 *(leu2 his4)*.

Expression and purification of recombinant proteins

Yeast transformation, cultivation and recombinant protein purification were carried out as described previously [8, 9, 10]. The His-tagged capsid protein was purified by nickel chelation chromatography [8, 9]. The non-His-tagged capsid protein was purified by CsCl gradient ultracentrifugation according to Samuel et al. [10]. Both proteins after purification were dialysed in PBS buffer. After dialysis the proteins were stored at – 20 °C prior to testing.

SDS-PAGE and Immunoblot

Protein samples were separated in 12% SDS-PAGE gels and transferred to Immobilon-P Polyvinylidene difluoride membrane (Millipore, Bedford, USA) by semi-dry blotting. After transfer the membrane was blocked with 5% dry milk in PBS containing 0.1% TWEEN-20 (PBS/T) for 1 h. The membrane was incubated for 2 h with RV-C specific monoclonal antibodies (Chemicon Europe Ltd, Harrow, UK) (diluted 1:1000 in PBS/T). Thereafter, the membrane was incubated with Anti-mouse IgG–HRP conjugate (Sigma-Aldrich, Steinheim, Germany) and diluted 1:1000 in PBS/T for 1 h. The membrane was stained by adding 4-chloro-naphthol supplemented with H_2O_2 (Sigma, Steinheim, Germany).

Proteins on SDS-PAGE gel were stained with Coomasie-blue R-250.

Electron microscopy

After purification with CsCl, centrifugation suspensions of RC protein were placed on 400-mesh carbon-coated palladium grids. Samples were stained with 2% aqueous uranyl acetate solution and examined with a JEM-100S electron microscope.

Indirect ELISA

Microtiter plates (Nerbe plus, Winsen/Luhe, Germany) were coated overnight at 4 °C with 100 µl yeast-derived rubella C protein at a concentration of 1µg/ml in 0.05M sodium carbonate buffer (pH 9.6). The plates were blocked with 3% bovine serum albumin in PBS. After the blocking and at each subsequent step the plates were washed three times with PBS-T. Thereafter, 100 µl of serum samples diluted 1:200 in PBS containing 0.05% TWEEN-20 with 1% BSA were added and incubated with antigen-coated wells for 2 h at 37 °C. Then 100 µl anti-human IgG – HRP conjugate (Sigma-Aldrich, Steinheim, Germany) diluted 1:7000 in PBS containing 0.05% TWEEN-20 were added to the wells and incubated for 1 h at 37 °C. The enzymatic reaction was developed by adding 100 µl of TMB substrate (3-, 3'-, 5-, 5'-tetramethylbenzidine) (Sigma, Steinheim, Germany) and stopped after 10 min of incubation at room temperature by adding 100 µl of 10% sulphuric acid. The optical density (OD) was measured at 450 nm on an automatic microplate reader (Tecan, Salzburg, Austria).

Human sera and sera pools

Human sera known to be positive or negative for anti-RV antibodies were received from Ethiopian Health Nutrition Research Institute (Addis Ababa, Ethiopia).

Determination of cutoff value

All the samples were tested in duplicate in two wells with and without antigen. The calculated OD value was well with antigen minus well without antigen. The cutoff value was estimated using seven negative samples. The mean absorbance of seven negative sera which were determined as negative with the Dade Behring Enzygnost Anti-rubella-virus / IgG Kit (Marburg, Germany) were calculated for each protein. The cutoff value was calculated as the average OD of negative samples plus 3 standard deviations.

All the values above the cutoff level were interpreted as positive and the lower values as negative.

RESULTS AND DISCUSSION

Synthesis of rubella capsid gene

The rubella virus genome has a G+C content of 69%, the highest of any RNA virus identified to date, which is associated with a high degree of secondary and tertiary structures of RNA that may impede the replication, transcription and translation of protein [11]. Rubella virus capsid gene was synthesized at the GenScript corporation. The gene has been optimized for *S. cerevisiae* expression by selecting codons optimal for yeast and to generate the gene with a lower G+C content. The sequence of the new gene is presented in Fig. 1. The optimized codons for the yeast expression system enhanced the expression level of

ATGGCTTCTACTACTCCAATTACTATGGAAGATTTGCAAAAAGCTTTGGAAGCTCAATCTAGAGCTTTGAGAGCTGAATTGGCTGCTG
GTGCTTCTCAATCTAGAAGACCAAGACCACCAAGACAAAGAGATTCTTCTACTTCTGGTGATGATTCTGGTAGAGATTCTGGTGGTCCAAGAAGAAGAAGAGGTA
ATAGAGGTAGAGGTCAAAGAAGAGATTGGTCTAGAGCTCCACCACCACCAGAAGAAAGACAAGAAACTAGATCTCAAACTCCAGCTCCAAA
ACCATCTAGAGCTCCACCACAACAACCACAACCACCAAGAATGCAAACTGGTAGAGGTGGTTCTGCTCCAAGACCAGAATTGGGT
CCACCAACTAATCCATTTCAAGCTGCTGTTGCTAGAGGTTTGAGACCACCATTGCATGATCCAGATACTGAAGCTCCAACTGAAGCTT
GTGTTACTTCTTGGTTGTGGTCTGAAGGTGAAGGTGCTGTTTTTTATAGAGTTGATTTGCATTTTACTAATTTGGGTACTCCACCATTGGATGAAGA
TGGTAGATGGGATCCAGCTTTGATGTATAACCCATGTGGTCCAGAACCACCAGCTCATGTTGTTAGAGCTTATAATCAACCAGCTGGTGATGTTAG
AGGTGTTTGGGGTAAAGGTGAAAGAACTTATGCTGAACAAGATTTTAGAGTTGGTGGTACTAGATGGCATAGATTGTTGAGAATGCCAGTTA
GAGGTTTGGATGGTGATTCTGCTCCATTGCCACCACATACTACTGAAAGAATTGAAACTAGATCTGCTAGAC
ATCCATGGAGAATTAGATTTGGTGCTCCACAAGCTTTTTTGGCTGGTTTGTTGTTGGCTACTGTTGCTGTTGGTACTGCTAGAGCTTAA

Fig. 1. Sequence of synthetic rubella virus capsid gene. Codons of translation initiation and termination are underlined

foreign protein. The sequence of the optimized gene has the G+C content of 43%.

Expression of rubella capsid protein in yeast cells

The synthetic rubella C gene was cloned into yeast expression vectors pFX7 and pFX7-6His resulting plasmids pFX7-RC and pFX7-6His-RC. SDS-PAGE of disrupted yeast cells, after induction with galactose, revealed in yeast extracts an intensive additional migrating band with a molecular weight of approximately 36 kDa consistent with the expected molecular weight [12] (Fig.2, A). Western blot with C-specific mAB (Chemicon Europe Ltd, Harrow, UK) detected two nearby molecular weight bands (Fig. 2 B). This is in agreement with the earlier findings of Waxham and Wolinsky [13] who determined that C fractionates with different pI values, most likely due to differences in phosphorylation [2, 14, 13]. Recombinant C protein from a pFX7-RC containing yeast was purified by ultracentrifugation in CsCl gradient. The recombinant protein forms a discrete band at the CsCl density 1.25–1.27 g/cm^3. The yield of recombinant C protein was approximately 3.2 mg of purified protein from 1 g of wet yeast biomass. EM analysis of these fractions demonstrated that the recombinant C protein was not forming any visible submolecular structures. 6His-tag containing the recombinant C protein was purified from pFX7-6His-RC-derived yeast by Ni-chelation technique under denaturing conditions according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The yield of the recombinant protein purified under denaturing conditions was similar to that obtained by using ultracentrifugation in CsCl gradient. Purification on Ni-chelate resin under native conditions was not successful because of a very low solubility of recombinant C protein. Therefore, the yield of the protein was significantly lower, and the protein was not used in the further experiments due to its high instability.

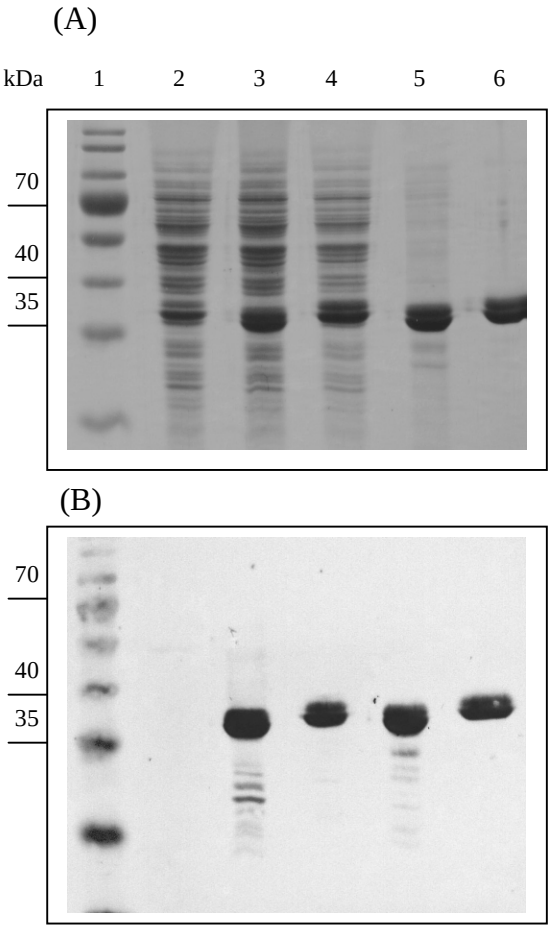

Fig. 2. Detection in yeast extracts and analysis of purified rubella C protein. (A) SDS-PAGE analysis. (B) Immunoblot with monoclonal antibodies specific to rubella C protein. Lane 1: protein molecular weight marker, Fermentas (Vilnius, Lithuania); lane 2: lysate of *S. cerevisiae* pFX7 transformed cells; lane 3: lysate of *S. cerevisiae* pFX7-RC transformed cells; lane 4: lysate of *S. cerevisiae* pFX7-6His-RC transformed cells; lane 5: CsCl-purified rubella C protein; lane 6: nickel-chelate purified His-tagged rubella C protein

The antigenic properties of different proteins were compared in an indirect IgG ELISA assay.

Antigenicity of recombinant rubella C proteins

The CsCl- and nickel-chelate-purified C proteins were evaluated for suitability as a diagnostic reagent. An indirect IgG ELISA test with rubella-negative and rubella-positive human sera was performed, in which CsCl-purified protein was compared with nickel-chelate--purified recombinant C protein. The results (Table) demonstrated differences in optical density at 450 nm between proteins purified under different conditions in indirect ELISA with rubella-positive serum specimens. ELISA tests with rubella-positive human serum samples revealed a high antigenicity of the CsCl-purified recombinant C protein which reacted efficiently with rubella-positive serum samples, while the reactivity of the corresponding protein purified under denaturing

Table. **Distribution of optical densities obtained for the indicated specimens in indirect ELISA using recombinant rubella C protein (RC) purified under different conditions** Cutoff values were: 0.041 for RC purified by CsCl ultracentrifugation, 0.039 for 6His-RC purified under denaturing conditions using nickel chelate chromatography, and 0.05 for 6His-RC purified by CsCl ultracentrifugation.

Antigen	A_{450} of rubella-negative sera pool	A_{450} of rubella-positive serum No. 1	A_{450} of rubella-positive serum No. 2	A_{450} of rubella-positive serum No. 3
1. RC (CsCl purified)	0.056	1.347	1.847	1.921
2. 6His-RC (purified under denaturing conditions)	0.052	0.172	0.123	0.195
3. 6His-RC (CsCl purified)	0.053	1.132	1.712	1.551

conditions was very weak (Table). The purified recombinant protein reacted very specifically, showing high optical density values in ELISA with rubella IgG-positive serum samples and no reactivity with rubella IgG-negative serum samples.

In order to eliminate the possible negative influence of 6His-tag on the antigenicity of C protein, 6-His-tagged protein was additionally purified by using ultracentrifugation in CsCl gradient. In this case, the antigenicity of both proteins purified in CsCl gradient was similar (Table). The results indicate that the antigenicity of C protein depends on the purification method. It seems likely that purification by CsCl ultracentrifugation does not disrupt protein conformation. In contrast, nickel chelate purification completely denaturizes protein, unfolds it and therefore reduces the antigenicity. Schmidt and co-workers also produced an antigenically active protein purifying the recombinant polyhistidine tagged rubella capsid protein under nondenaturing conditions [15].

Our study suggests that recombinant capsid protein purified by ultracentrifugation in CsCl gradient may serve as a useful reagent for rubella serology.

ACKNOWLEDGEMENTS

We are very grateful to Dr. Aurelija Žvirbliene for helpful discussions and for editing the manuscript. This work was supported by the Lithuanian State Science and Studies Foundation (grant No. B-11/2003–2006).

Received 14 February 2006
Accepted 23 June 2006

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RAUDONUKĖS VIRUSO KAPSIDĖS BALTYMO SINTEZĖ MIELĖSE IR JO CHARAKTERIZAVIMAS

S a n t r a u k a

Raudonukės viruso kapsidės baltymo genas buvo klonuotas į mielių ekspresijos vektorių ir ekspresuotas mielėse *S. cerevisiae*. Raudonukės virioną sudaro trys struktūriniai baltymai: kapsidės baltymas (C) ir du paviršiaus baltymai – E1 ir E2 glikoproteinai. Virusinės infekcijos metu yra indukuojama IgG, IgM ir IgA imunoglobulinų gamyba. Šių imunoglobulinų nustatymas gali būti panaudojamas raudonukės virusinės infekcijos diagnostikai.

Darbo tikslas buvo susintetinti raudonukės viruso kapsidės baltymą mielėse, įvertinti gauto rekombinantinio baltymo antigenines savybes ir galimybę jį panaudoti diagnostikai. Raudonukės viruso genomą sudaro didelė G + C sudėtis (69%), didžiausia tarp visų žinomų RNR virusų. Tokių genų ekspresija heterologinėse sistemose būna labai bloga dėl neefektyvios informacinės RNR transliacijos. Mūsų eksperimentuose buvo naudojamas sintetinis kapsidės baltymo genas su mielėms būdingais kodonais, kuriame G + C sudėtis buvo sumažinta iki 43%. Tokio geno ekspresija mielėse buvo labai efektyvi, rekombinantinio baltymo išeiga sudarė apie 3,2 mg viename grame drėgnos mielių biomasės svorio. Rekombinantinio baltymo gryninimui buvo naudojami du skirtingi metodai – centrifugavimas CsCl gradiente ir nikelio chelatinė chromatografija. Palygintos skirtingais metodais išgryninto rekombinantinio baltymo antigeninės savybės naudojant raudonukės virusu infekuotų žmonių kraujo serumo pavyzdžius. Nustatyta, kad baltymo, išgryninto CsCl gradiente, antigeniškumas yra didesnis. Tai rodo, kad šis rekombinantinis baltymas gali būti tinkamas raudonukės viruso diagnostikai.