Synthesis of human mumps virus nucleocapsid protein in yeast *Pichia pastoris*

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During the past few decades, the yeast *Saccharomyces* has become an attractive host for the production of recombinant proteins. Methylotrophic yeasts represent an attractive alternative due to strong, tightly regulated promoter elements. Moreover, an advanced fermentation technology has been developed for yeast production from methanol. To develop improved reagents for mumps virus serology, a high-level yeast *Pichia pastoris* expression system was employed for production of recombinant mumps nucleocapsid protein. The nucleocapsid protein was purified by CsCl gradient centrifugation and yielded approximately 50 mg/l of yeast culture. The electrophoretic mobility in SDS-PAGE for native and yeast-derived nucleocapsid protein was similar to that of native nucleocapsid protein in SDS-PAGE. In this study, we have demonstrated that the yeast *P. pastoris* is an excellent host for a high-level production of mumps virus nucleocapsid protein.

Key words: expression, mumps virus, nucleocapsid protein, *Pichia pastoris*

INTRODUCTION

Mumps virus contains a negative sense, single stranded RNR virus and belongs to the family *Paramyxoviridae*. The virus contains six major structural proteins: nucleocapsid associate protein (NP), phosphoprotein (P), matrix (M), small hydrophobic (SH), two surface glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F) protein, and a negligible amount of large (L) protein associated with NP. The function of NP has appeared to be in charge of viral genome packaging and formation of replication complexes along with the other proteins. Viral nucleocapsid proteins usually elicit a strong long-term humoral immune response in patients as well as in experimental animals. Therefore, immunoassays for detection of antibodies specific to mumps NP have formed the basis for mumps diagnosis [1, 2]. Yeasts are unicellular eucaryotic microorganisms, in which the ease of genetic manipulations can be combined with the ability to perform eucaryotic processing steps on the polypeptides expressed and thus to constitute attractive systems for various aspects of modern biotechnology [3]. Our aim was directed towards construction of efficient yeast producers for generation of nucleocapsid protein for mumps serology. In this paper we describe an NP expression study in the commercially available methylotropic yeast *Pichia pastoris* system.

MATERIALS AND METHODS

Strains, media and transformation. Bacterial recombinants were screened in *E. coli* DH5αF' cells. The strain *P. pastoris* GS115 his4 (Invitrogen) was used in yeast expression experiments. Transformation of *P. pastoris* GS115 his4 was performed by electroporation according to Cregg [4]. *P. pastoris* His^+ transformants were selected on minimal agar medium (1.34% YNB, 1% glycerol).

Cloning of mumps virus NP gene into yeast vector and expression in yeast. All DNA manipulations were performed according to standard procedures [5]. Enzymes and kits for DNA manipulations were purchased from Fermentas AB (Vilnius, Lithuania). The mumps virus NP gene was amplified by PCR from cDNA of the Gloucester strain [2]. The primers used for amplification incorporated the *Xba*I site for subcloning into *P. pastoris* vector pPIC3.5 (Invitrogen). The resulting plasmid pPIC3.5-NP was linearized and used for electroporation into *P. pastoris* GS115 [5]. Multicopy recombinant transformants were selected according to Romanos [6]. The selected *P. pastoris* GS115 [pPIC3.5-NP] transformants, which contained integrated ~10 copies of

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mumps NP gene under control of *AOX1* promoter and transcription terminator, were inoculated into 250 ml of BMG medium (100 mM potassium phosphate, pH 6, 1.34% YNB, 4 × 10^{-5}% biotin, 1% glycerol) in a 2.5 l flask. For induction, the cells were resuspended in BMM (100 mM potassium phosphate, pH 6, 1.34% YNB, 4 €10^{-5}% biotin, 1% methanol) medium to 2 OD_{600}, and for induction the culture was supplemented with methanol at 6 h intervals. After induction for 48 h the cells were pelleted and stored at –70 °C [7]. 10 g of yeast cells biomass was thawed and disrupted as described by Samuel et al. [2]. The suspension after disruption was diluted by adding 10 ml of disruption buffer, homogenized and centrifuged for 10 min at 3000 g and at 4 °C. 15 ml of supernatant was collected and loaded onto a chilled 10 ml 30% sucrose cushion in centrifuge tubes and ultracentrifuged at 100000 g for 3 h at 4 °C. The supernatant was discarded and the pellet once again re-suspended in a small volume (~2 ml) of the disruption buffer and was then loaded on CsCl gradient ranging from 1.23 g/ml to 1.38 g/ml and centrifuged for 48 h at 100000 g for 48 h (Beckman, 60 Ti). The fractions were collected and those containing NP were dialysed against PBS for further analysis [2]. The buoyant density of the fractions was determined with a refractometer. Monoclonal antibody and horseradish peroxidase (HRP)-labelled anti-mouse IgG conjugates (Dako, U. K.) were used for detection of mumps NP.

RESULTS AND DISCUSSION

Synthesis of mumps NP in yeast *P. pastoris*. Previously we have demonstrated an efficient generation of mumps NP nucleocapsid-like particles (NLPs) in the the yeast *S. cerevisiae* [2]. In the present paper we analysed mumps NP synthesis in the methylotrophic yeast *P. pastoris*. The mumps NP gene was inserted in the plasmid pPIC3.5 under control of the methanol-inducible *AOX1* promoter to give the plasmid pPIC3.5-NP. SDS-PAGE analysis of *P. pastoris* containing a high copy number of integrated pPIC3.5-NP cassettes (~10 copies) after growth in methanol medium revealed an additional band of the molecular size corresponding to NP (66 kDa) (Figure, lane 3). No bands of such molecular size were observed in control strains of *P. pastoris*, harboring the integrated vector pPIC3.5 or *P. pastoris* [pPIC3.5-NP] before induction (Figure, lane 2). Immunostaining with monoclonal NP specific antibodies confirmed that the additional bands of 66 kDa observed in yeast lysates belonged to mumps NP protein (Figure, lanes 6).

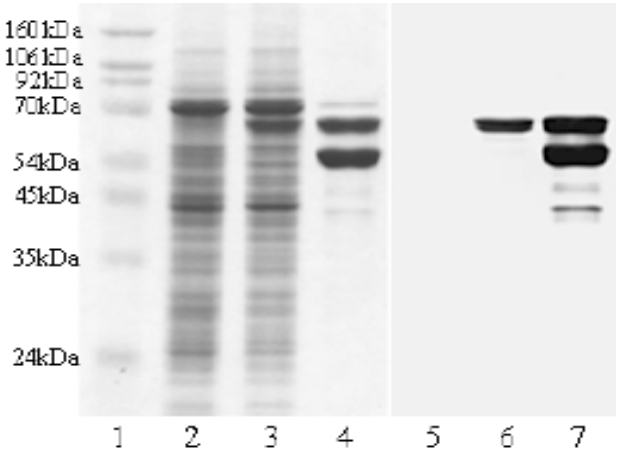

Figure. SDS-PAGE and immunoblot analysis of mumps NP. Lane 1 – prestained protein molecular weight markers (AB Fermentas, Lithuania); lane 2 – *P. pastoris* [pPIC3.5-NP] in growth medium; lane 3 – *P. pastoris* [pPIC3.5-NP] in induction medium; lane 4 – CsCl centrifugation purified mumps NP; lane 5 – immunoblot of *P. pastoris* [pPIC3.5-NP] in growth medium; lane 6 – immunoblot of *P. pastoris* [pPIC3.5-NP] in induction medium; lane 7 – immunoblot of CsCl centrifugation purified mumps NP

Purification of yeast-derived mumps NP in CsCl gradient. To study the capacity of self-assembly of the expressed NP derivatives in the yeast *P. pastoris,* cell lysates were analysed after centrifugation through 30% sucrose. In the sediments nucleocapsid protein was found (data not shown). Sedimentation of recombinant proteins through a 30% sucrose cushion confirmed a large multimeric organization of recombinant protein [2]. Further ultracentrifugation in the CsCl gradient and fraction analysis revealed a buoyant density of 1.29–1.30 g/ml characteristic of most virus-like particles (VLPs) or nucleocapsid-like particles (NLPs) [8, 9]. SDS-PAGE analysis of CsCl purified protein revealed bands of 66 kDa (Figure, lanes 4) corresponding to those identified in crude lysates (Figure, lane 3). However, it has been observed that purification of NP from yeast leads to protein degradation. The band that dominated for NP derived from *P. pastoris* corresponded to 52 kDa (Figure, lane 4). Such NP protein degradation was described earlier during purification of NP protein from mumps-virus-infected cells, and additional polypeptide bands of 52 kDa and 45 kDa were observed [9]. In several preparative purification procedures of NP, the yield of CsCl gradient purified NP was approximately 50 mg per 1 l of *P. pastoris* cell culture or 2.1 mg of NP per 1 g wet biomass. In this study we have demonstrated that yeast *P. pastoris* is an excellent host for a high-level production of mumps virus NP.

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KIAULYTĖS VIRUSO NUKLEOKAPSIDO BALTYMO SINTEZĖ *PICHIA PASTORIS* MIELĖSE

S a n t r a u k a

Pastaraisiais dešimtmečiais mielės tapo vienu iš populiariausių rekombinantinių baltymų producentų. Iš visų mielių rūšių, metilotrofinės mielės *Pichia pastoris* išsiskiria labai stipriais ir patikimai reguliuojamais promotoriais. Šiame darbe mes ištyrėme galimybę *P. pastoris* mielėse sintetinti žmogaus kiaulytės (parotito) viruso nukleokapsido baltymą. Valyto baltymo išeiga sudarė apie 50 mg iš vieno litro mielių kultūros. Viruso baltymas naudojamas naujoms, jautrioms ir greitoms viruso testavimo sistemoms sukurti.