Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Biotechnology 120 (2005) 347–359
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Production of bifunctional proteins by *Aspergillus awamori*: Llama variable heavy chain antibody fragment (V_{HH}) R9 coupled to *Arthromyces ramosus* peroxidase (ARP)

Vivi Joosten [a,*,1], Marc S. Roelofs [a,b], Niels van den Dries [a], Theo Goosen [a], C. Theo Verrips [b], Cees A.M.J.J. van den Hondel [a,c], B. Christien Lokman [a,2]

[a] *TNO Quality of Life, Department of Microbiology, Utrechtseweg 48, P.O.Box 360, 3700 AJ Zeist, The Netherlands*
[b] *Department of Molecular Cell Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[c] *Clusius Laboratory, Fungal Genetics Research Group, Institute of Biology, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands*

Received 9 February 2005; received in revised form 2 June 2005; accepted 21 June 2005

Abstract

The *Arthromyces ramosus* peroxidase gene (*arp*) was genetically fused to either the 5′- or 3′-terminal ends of the gene encoding llama variable heavy chain antibody fragment V_{HH} R9, resulting in the fusion expression cassettes ARP-R9 or R9-ARP. *Aspergillus awamori* transformants were obtained which produced up to 30 mg l^{-1} fusion protein in the culture medium. Both fusion proteins showed peroxidase activity in an ABTS activity test. Considerable amounts of fusion protein were detected intracellularly, suggesting that the fungus encounters problems in secreting these kind of proteins. ELISA experiments showed that ARP-R9 was less able to bind its antigen, the azo-dye RR6, as compared to R9-ARP. Furthermore, in contrast to R9-ARP, ARP-R9 bound to RR6 did not show peroxidase activity anymore. These results indicate that fusion of ARP to the C-terminus of the antibody fragment V_{HH} R9 (R9-ARP) is the preferred orientation.

Keywords: Llama variable heavy chain antibody fragments (V_{HH}s); *Arthromyces ramosus* peroxidase (ARP); "Magic Bullets"; Fusion proteins; *Aspergillus awamori* ;Heterologous protein production

1. Introduction

Genetically coupling of enzymes and antibodies (in this manuscript referred to as bifunctional proteins or fusion proteins) allows interesting applications due to the fact that antibodies can direct the coupled enzyme to the place where it should perform its enzymatic

* Corresponding author.
E-mail address: vivi.joosten@wur.nl (V. Joosten).
[1] Present address: Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[2] Present address: HAN University, P.O. Box 31272, 6503 CG Nijmegen, The Netherlands.

0168-1656/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2005.06.034

function. This can result in an enhanced efficiency for the usage of the enzyme, since it is brought closer to the site of action and also less of the protein is required. This so-called "Magic Bullet" approach has already been developed in the medical field where for example anti-tumour agents are directed towards the site of malignant growing cells by means of antibodies that recognise these cells (Hudson, 1998; Reiter, 2001; von Mehren and Weiner, 1996). The use of bifunctional proteins in consumer products like detergents, toothpastes and shampoos is a less exploited area, but with the help of modern biotechnology the applicability of these molecules is within reach (Szynol et al., 2004; reviewed in Joosten et al., 2003). We are interested in the application of bifunctional proteins for the improvement of detergents. Enzymes, like peroxidases that are able to bleach persistent spots on laundry, can be directed to these spots by coupling them to specific antibodies (Beggs et al., 1998). The idea is that the enzyme-part of the fusion protein will be enriched at the target site, and therefore, mainly act at the spot. This would leave the "clean" textile untouched, which is believed to be less harmful for the textile. The generation of detergents capable of removing difficult spots by the addition of bifunctional proteins will result in the use of less chemical bleaching components in detergents, thereby diminishing environmental pollution.

For the application of antibodies in detergents they should be stable under the harsh conditions of laundry washing and simple in structure to permit their production by relevant industrial micro-organisms. Hamers-Casterman et al. (1993) discovered a novel class of IgG antibodies in *Camelidae* (camels and llamas) that are devoid of light chains. Their binding domains consist only of the heavy chain variable domains, called V_{HH}s (Muyldermans et al., 1994). V_{HH}s are of great interest since they comprise the smallest possible recognition units of antibodies (Sheriff and Constantine, 1996) and are very simple in structure. More importantly, V_{HH}s were shown to be highly soluble and very stable at high temperatures (Ghahroudi et al., 1997; van der Linden et al., 1999). Denaturation of V_{HH}s through unfolding was shown to be a reversible process, mainly due to the absence of required V_H/V_L association, present in conventional antibodies or fragments thereof (Perez et al., 2001).

One of the peroxidases that is of major interest for improvement of detergents is the fungal *Arthromyces ramosus* peroxidase (ARP; Akimoto et al., 1990; Sawai-Hatanaka et al., 1995). Peroxidases are enzymes that utilise hydrogen peroxide to catalyse the oxidation of a wide range of organic and inorganic compounds and are produced by a variety of microbial organisms (in particular fungi), plants and animals. ARP is a 41 kD monomeric glycoprotein that contains protoheme IX as a prosthetic group. It has a broad specificity for phenolic and anilinic substrates that make the enzyme suitable for usage in bleaching processes (Kjalke et al., 1992).

The applicability of using bifunctional proteins in consumer application requires that the proteins can be produced cheaply, in large amounts and that the final product can be isolated easily from the culture medium. Filamentous fungi have the capability of secreting large amounts of protein into their culture medium. Therefore, these organisms are widely used for the production of commercially interesting proteins (reviewed by Punt et al., 2002; van den Hondel et al., 1991; Verdoes et al., 1995). Especially species from the genus *Aspergillus* are attractive candidates for the production of fusion proteins, comprising ARP fused to a llama heavy chain antibody fragment (V_{HH}). Recently, we have demonstrated that in *A. awamori* under control of the 1,4-β-endoxylanase A (*exlA*) promoter (Gouka et al., 1996a) secretion of active ARP was achieved up to 800 mg l^{-1} in shake-flask cultures (Lokman et al., 2003). In a previous report we also showed that functional V_{HH}s raised against the azo-dye RR6 could be produced by *A. awamori* (Joosten et al., 2005).

In this paper, we studied the production of bifunctional proteins by *A. awamori*. For this purpose, *A. ramosus* peroxidase was genetically fused to heavy chain antibody fragment V_{HH} R9. Produced V_{HH}-ARP fusion proteins were tested in assays to determine the preferred orientation (N-terminal or C-terminal linkage to the antibody fragment) for potential applications of these "Magic Bullets" in detergents.

2. Materials and methods

2.1. Strains and media

A. awamori pyrG$^-$ mutant strain AWC4.20 (Gouka et al., 2001), used as a recipient for transformation, is a derivative of *A. awamori* ATCC 11358 (CBS 115.52).