Bacterial Luciferase of *Vibrio harveyi* MAV: Purification, Characterization, and Application

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Summary. In the medical and biotechnological industry is an increasing interest for bioluminescent systems for example as analytical tools and genetic markers. We introduced a new procedure for a large scale purification of luciferase from *Vibrio harveyi* MAV in view of subsequent crystallization of the enzyme. In four steps the luciferase was obtained in greater than 99 % purity using the chromatography equipment (FPLC-System) from Pharmacia/LKB, Freiburg, Germany. Homogeneity of the luciferase was proved by SDS-PAGE and silver staining. The enzyme was purified 34fold to a yield of 29 % and a specific activity of $1.8*10^{11}$ [LU/mg]. The optimum activity of luciferase was detected at pH 6.8 and 30°C. The isoelectric point proved to be at pH 4.7. Treatment with the inhibitors diethylcarbonate, phenylmethylsulfonylfluoride, and diethyl-p-nitrophenylphosphate until unknown as luciferase inhibitors, affected the activity with the same extent and nearly the same velocity as other inhibitors, reacting with histidine and cysteine residues at the active center of luciferase. The enzyme crystallized depending upon different precipitation agents in various forms. We obtained two types of crystals, needles in ammonium sulfite and rhombic-like in ammonium sulfate.

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Introduction

In recent years applications for bioluminescent emission with simple photon-counting instrumentation increased dramatically. The reasons of taking bioluminescent systems for analytical purposes are their selectivity and high sensitivity. One of the most useful systems is the luciferase-oxidoreductase enzyme couple (Nabi A et al. 1986). The luciferase [alkanal monooxygenase (FMN-linked); alkanal, reduced-FMN: oxygen oxidoreductase (1-hydroxylating, luminescing), EC 1.14.14.3] from the marine bioluminescent bacterium *Vibrio harveyi* MAV catalyzes the the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aldehyde (decanal) in the presence of molecular oxygen with the concomitant emission of blue light (λ_{max} = 490 nm)(Escher A et al. 1989; Ziegler MM et al 1981):

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + h\nu$$

In combination with NAD(P)H-FMN oxidoreductase this bienzymatic system is an important diagnostic-analytical tool for the monitoring of medical tests or biotechnological processes. Any reaction that leads to the production or consumption of NAD(P)H can be measured by this light-emitting system. The commercially available bacterial luciferase (McCapra F. 1987) contains the necessary NAD(P)H-FMN oxidoreductase in varying amounts but contaminations by other enzymes severely limits its application e.g. in biosensor technique. Therefore several workers (Kurkijärvi K et al. 1982; Blum LJ et al. 1989; Girotti G et al. 1990) tried to increase the stability by enzyme immobilisation or whole cells that harbour the bioluminescent system. We elaborated a modified purification protocol (Hastings JW et al. 1978) for bacterial luciferase in view of achieving highest enzyme purity. The subsequent crystallization is the crucial step in the elucidation of the three-dimensional protein structure. With the aid of the luciferase structure it may be possible to design new specifities for technological applications (protein engineering), and to increase stability of the enzyme to prolong the life time of a biosensor.

Results

PURIFICATION OF THE LUCIFERASE. The bacterial strain used was the wild type of *Vibrio harveyi* MAV (Baumann et al. 1980). Luciferase activity in vivo and in vitro was measured using a batch luminometer (Berthold Biolumat LB 9501, Wildbad, Germany). Activity in vivo was taken as the intensity of light emitted from 1 ml culture in a vial. Activity in vitro was determined using either a modified standard flavin injection assay (Nabi et al. 1986) or a dithionite assay (Toyoba LTD 1990). Activity measurements were recorded as light intensity peaks over a period of 10 sec (light unit (LU) of quanta sec^{-1} (Hastings JW et al. 1978)). Calibration of the instrument was followed with a radioactive standard (Hastings JW et al. 1963). Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the PhastSystem, ready-to-use gels (separating gel: 8-25 % T) and SDS-buffer strips from Pharmacia/LKB according to the PhastSystem separation technique file No. 110. The gels were stained with $AgNO_3$ according to the PhastSystem development technique file No. 210 (Heukeshoven et al. 1985).

Vibrio harveyi was incubated in 300 l of modified artificial sea salt medium (Utlizur S. 1979) in a 350 l fermentor (Giovanola Freres S.A. Monthey, Suisse) at 30°C under aeration at 0.5 VVM (volume air per volume medium per minute) and 600 rpm (rounds per minute). The pH was maintained between 6.6 -7.0 by the addition of NaOH. During the incubation cell density and bioluminescence were monitored in samples removed every 30 min from the fermentor in order to finish cultivation if the mid-log phase of the bacterial growth curve was reached (3 hours respectively optical density 660 nm = 1.5). The yield of packed wet cells was 5.5 g from 1 l of liquid medium.

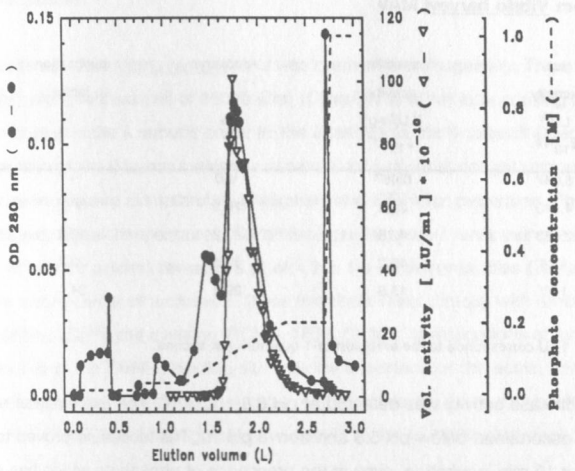

Fig. 1: Elution pattern from adsorption chromatography of MAV luciferase on hydroxyapatite column. Elution was carried out with a phosphate gradient, pH 6.3 (see text).

All purification steps were performed at 4°C. Frozen cells were thawed overnight, mixed 1:1 (w/v) with cold buffer (Colepicolo P. et al. 1989) and lysed osmotically by continuous stirring. Thirty minutes were required for complete lysis. Cellular debris was removed by centrifugation at 18000 rpm in a Beckman centrifuge. The supernatant was loaded on two coupled ion exchange chromatography columns, a cation exchange, 500 ml S-Sepharose FF (ϕ 5x25cm) as first and an anion exchange column, 750 ml Q-Sepharose FF (ϕ 6x25cm) as second. A large amount of protein was retained by the S-Sepharose FF, while the luciferase was passed through and bound to Q-Sepharose FF. In a stepwise gradient of phosphate the luciferase was eluted from the matrix at 0.3 [M]. The combined active fractions of the anion exchange chromatography were mixed with 1 M $(NH_4)_2SO_4$ by continuous stirring. The solution was applied to a hydrophobic interaction chromatography column (270 ml Phenyl-Sepharose CL-4B (ϕ 5x13.5cm)). The luciferase eluted by a linear gradient of 1 M - 0 M $(NH_4)_2SO_4$ (4 l) between 0.5 and 0.0 [M] $(NH_4)_2SO_4$. After pooling the active fractions they were concentrated 6 times using a cross-flow ultrafiltration membrane (type omega, Filtron) with a nominal molecular weight cut-off of 10 kDa.

The ultrafiltration retentate was loaded onto a hydroxyapatite column ((110 ml, ϕ 2.5x22cm) adsorption chromatography). After washing the column the luciferase was eluted by a double stepwise gradient; 1st: 10 mM - 30 mM (0.1 l), 2nd: 30 mM - 200 mM (1.5 l), 3rd: 0.2 M - 1 M phosphate (0.1 l) (Fig. 1). Enzyme containing fractions were collected, concentrated 3 times by ultrafiltration (see below) and applied to an affinity chromatography column (100 ml ω-Aminohexylagarose (ϕ 2.5 x20cm)). The enzyme was eluted by a stepwise gradient; 1st: 25 mM - 150 mM (1000 ml), 2nd: 0.15 M -1 M phosphate (50 ml) (Fig. 2),(Table 1).

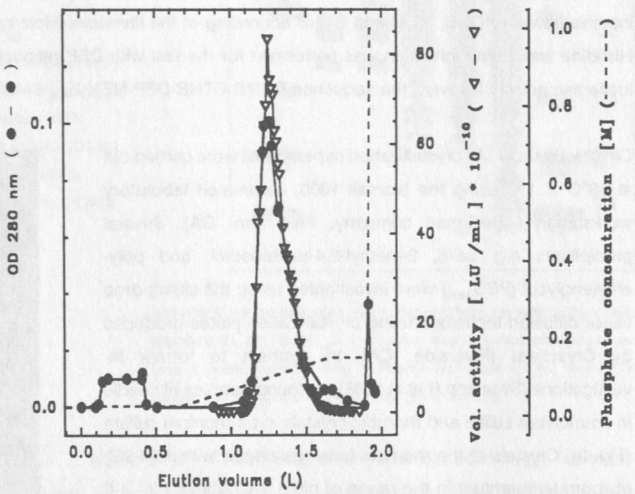

Fig. 2: Elution profile from affinity chromatography of MAV luciferase on ω-Aminohexylagarose. Elution was carried out with a phosphate gradient, pH 6.3 (see text).

Table 1: Purification of luciferase from Vibrio harveyi MAV

step	tot. protein (mg)	tot. activity LU[a] $*10^{-14}$	specific activity [LU/mg] $*10^{-11}$	recovery %	purification factor
crude extract	13279	6,047	0,457	100	-
Q-Sepharose FF	1434,6	4,810	3,35	79,2	7,3
Phenyl-Sepharose Cl-4B	601,0	2,863	4,76	47,2	10,3
Hydroxyapatite	181,4	1,84	10,2	30,5	22,2
Ω-Aminohexylagarose	113,4	1,77	15,6	29,2	34,4

a) luciferase activity was determined as light units (LU). 1 LU corresponds to the emission of 1 quantum per second.

ENZYMATIC CHARACTERIZATION. The optimum luciferase activity was detected at pH 6.8 and 30°C. The examination of pH stability revealed that no activity could be ascertained below pH 5.5 and above pH 10. The luciferase proved to be instable at temperatures of above 40°C after 10 min incubation, even in the prescence of phosphate which has a stabilizing effect on luciferase (Holzman TF et al 1980). Investigations of substrate specifity of luciferase carried out with the standard assay at 30°C showed some differences of former examinations (Meighen EA et al 1971; Holzman TF et al 1983). The luciferase possessed a high affinity towards n-decanal. The enzyme had a K_M of 18.92 nM ± 0.5. Above 21 nM aldehyde the contribution of substrate was reversible inhibited. The K_M for $FMNH_2$ was 1.64 μM ± 0.3. For measurements of the aldehyde specifity of luciferase aliphatic compounds of the chain length C_8, C_{10}, C_{11}, C_{12} and C_{14} were used. Aliphatic aldehydes in the sequence of carbon length 8-11-12-14-10 reacted with an increasing activity with the enzyme. Luciferase (17.5 μM final concentration) in 868 μl phosphate buffer (0.1 M, pH 7) was incubated at room temperature with different inhibitors (46 μl) (170 μM final concentration). We found that the luciferase was inhibited within the first 15 min of incubation by investigated inhibitors. The cysteine residues were inactivated with PCMB, NEM and DTNB according of the literature (Holzman TF et al 1981; Ziegler MM et al 1981). Histidine and serine inhibition was performed for the first with DEP respectively PMSF and E_{600}. The inactivation of luciferase activity followed the sequence PCMB-DTNB-DEP-NEM-E_{600}-PMSF.

CRYSTALLIZATION . All crystallization experiments were carried out at 18°C ± 1°C using the biomek 1000, automated laboratory workstation (Beckman company, Palo Alto, GA). Several precipitants e.g. salts, 2-methyl-2,4-pentanediol, and polyethylenglycol (PEG_{6000}) were investigated using the sitting-drop vapor diffusion technique using crystallization plates produced by Cryschem (Riverside, CA). In contrast to former investigations (Swanson R et al 1985) we found bundles of needle in ammonium sulfite and rhombic crystals in ammonium sulfate (Fig. 3). Crystals of the rhombic form developed within 24-96h at room temperature in the range of pH 6.5 - 7.5 and 1.3 - 1.6 [M] $(NH_4)_2SO_4$.

Fig. 3: Crystal of native luciferase from *Vibrio harveyi* MAV; 1.3 M $(NH_4)_2SO_4$, 9.6 mg protein / ml, pH 6.6 (150 x 250 μm).

Discussion

Luciferase from Vibrio harveyi MAV was purified to homogeneity. The enzyme is composed of one α subunit of 43000 [Da] and one ß subunit of 35000 [Da] (Cline TW et al 1972) according to SDS-PAGE (Fig. 4, Table 2). The active site is located in the α subunit close to the interface to the ß subunit (Ziegler MM et al 1986; Chen LH et al 1989). The function of the ß subunit which is essential for activity is not yet understood. Further examinations showed, that the enzyme requires Dithiothreitol, phosphate and EDTA for protection. Phosphate enhanced the stability of the enzyme towards higher temperatures. Chromatofocusing experiments using the equipment of the FPLC-System with a Mono P HR (5/20) column revealed a pI of 4.7 ± 0.1. Inhibitor studies (Table 2) display the involvement of amino acids in the active center of luciferase. These inhibitors react directly with amino acid residues including serine (PMSF,E_{600}), histidine (DEP) and cysteine (PCMB, NEM, DTNB). Investigations of further inhibition studies releaved that a cysteine residue at the $FMNH_2$ binding site may be important for the active center of luciferase.
More structural information could be received through X-ray analysis from crystals of bacteriell luciferase. This leads to the possibility of "designing" new specifities for technological applications (protein engineering).

Table 2: Some properties of bacterial luciferase

Molecular weight	85000 ±2100
α subunit	43242 ±1428
ß subunit	34679 ±1040
Isoelectric point	4,7 ± 0,1
pH optimum	6,8
Temperature optimum	30°C
Temperature stability	20-40°C
pH stability	6-9 (6h 30min,30°C)
Inhibitors	phenylmethylsulfonylfluoride (PMSF), diethyl-p-nitrophenylphosphate (E_{600}), diethylpyrocarbonate (DEP), 5,5'-dithiobis-2-nitrobenzoate (DTNB), N-ethylmaleimide (NEM), p-hydroxymercuribenzoate (PCMB)

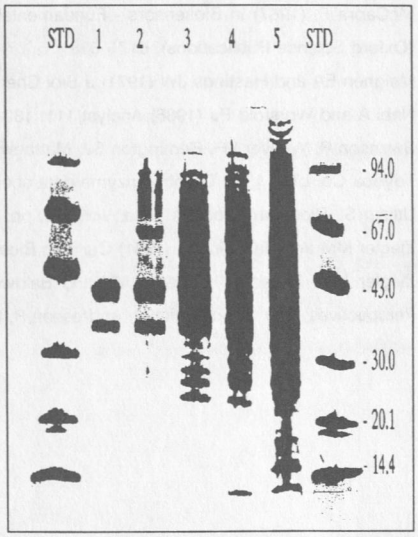

Fig. 4:
SDS-PAGE of samples from Vibrio harveyi: (from the left) STD: protein standard 91, 67, 43, 30, 20.1, 14.4 kd; lane 1: Ω-Aminohexylagarose; lane 2: hydroxyapatite; lane 3: Phenyl-Sepharose CL-4B; lane 4: Q-Sepharose FF; lane 5 crude extract; silver staining.

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