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SPECIFIC PURIFICATION OF ALCOHOL DEHYDROGENASE FROM *Saccharomyces cerevisiae;* QUALITATIVE AND QUANTITATIVE CHARACTERIZATION

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ABSTRACT

Objectives: This experiment was conducted to rule out unspecific proteins and enzymes concomitantly extracted during alcohol dehydrogenase enzyme (ADH) purification process in *Saccharomyces cerevisiae* using affinity chromatography.

Method: Cell disruption was performed and the ADH was treated by desalting followed by isolation and purification by affinity chromatography. For the control of the individual

purification steps, the enzyme activity and the total protein concentration were measured photometrically. By means of these two values, the specific enzyme activity could be calculated for a statement of the purification quality.

Results: The overall purification of ADH was confirmed by the SDS-PAGE visualizing the purification steps. Additionally, the protein size of ADH was determined by comparison with a protein ladder and an ADH standard and confirmed by affinity chromatography. The graphical comparison of values showed a decrease in the enzyme activity and the total protein concentration for all purification steps except for ADH. This is plausible since all proteins except for ADH should be removed during the purification, which causes a lower signal. However, the specific enzyme activity of ADH increased during isolation. Eventually, the desalting of samples resulted in an increase estimated 125% $p<0.05$.

Conclusion: The desalting treatment step is highly recommended in the process of specific ADH enzyme purification using affinity chromatography.

Keywords: ADH, Protein purification, Affinity chromatography, Enzyme activity, *Saccharomyces cerevisiae*

INTRODUCTION

Saccharomyces cerevisiae, the ancestor of the human pathogen *Candida albicans*, is the most abundant eukaryotic microorganism utilized in the food industry [1 , 2]. Alcohol dehydrogenase (ADH) is a fundamental enzyme in ethanol metabolism. ADH catalysis the oxidation of alcohol to aldehyde and ketone in the presence of the coenzyme nicotinamide adenine dinucleotide (NAD^+). ADH is commonly present in many organisms such as humans, animals, yeast, bacteria or plants. Yeast ADH can also catalyze the back reaction from acetaldehyde to ethanol during the fermentation of glucose in the absence of oxygen [3, 4]. The importance of ADH is attributed significantly to the capability of ethanolic fermentation of carbohydrate feedstocks [5].

The ADH enzyme family comprises at least three distinct enzyme superfamilies: iron-activated alcohol dehydrogenases, medium-, and chain and short-chain dehydrogenases/ reductases [6, 7]. There are seven isoforms of ADHin the universe; the abundant form is the homotetramers which subsist of identical four protein subunits. Each subunit interacts consequently with a structural element, a catalytic unite and two zinc ions, whereas, other ADH types are homodimers interact with only two subunits [7, 8]. Because of the

importance of ADH enzyme in the industry several research groups have studied and described the purification process of ADH using different techniques; for instance, using Fast Protein Liquid Chromatography (FPLC) or Size Exclusion Chromatography [9-12]. FPLC used for the purification of active molecules as separation of proteins and nucleotides at lower pressure, whereas, Size Exclusion Chromatographyis adopting the molecular size in separation [13]. To achieve a high outcome yield with a good quality of ADH enzyme; different steps of exudate treatment should be performed before injecting the sample in the FPLC. Firstly, there should be a process of cell wall disruption either by mechanical forces or by enzymatic digestion or even using a lysis buffer [14]. Secondly, the process of removing undesired cellular macromolecules including polysaccharides, lipids, and nucleic acids either by extraction process or desalting and protein precipitation [10].

In this work, we will try to rule out unspecific proteins and enzymes concomitantly extracted during alcohol dehydrogenase extraction in *Saccharomyces cerevisiae* and to characterize the effect of treatment on the quality and quantity of the purification process.

MATERIAL AND METHODS

The experiment was performed as described in the script [15] except Coomassie Brilliant Blue was used as a dye for staining the proteins on the SDS-PAGE.

METHODS

Yeast culture

Saccharomyces cerevisiae (strain ATCC 204508 / S288c) cells were cultivated in 500 ml of synthetic culture medium (10 g glucose, 10 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 2 g yeast extract, 0.8 g MgSO4,) (Sigma). Three ml of freshly prepared yeast cell suspension (10^8CFU/ml) in the exponential log phase was added to the vessel. The incubation temperature was adjusted to 30 °C with a continuous stirring rate of 200 rpm for the culture medium. The cells were harvested at an A of 4.3, resulting in 3.25 g, later on, yeast cells were resuspended in one volume of buffer A (20 mM Tris/HCl, pH 8.0, 2mM dithiothreitol).

Homogenization in a Bead Mill

The procedure was executed according to the procedure described by Kath and Kulicke [16] with some modifications. The crude extract was disintegrated with the use of glass beads of 0.5 mm diameter on a bead-595 beater (Bead-Beater type GB26 homogenizer, Biospec Products) with the power of 400 W. The mill tank had a total

working volume of around 300 mL. According to the manufacturer's instructions, the load of beads should be in the range of minimally 50% and maximally 75% of tank volume. The beads were added to the volume of 180 cm3, which constituted 61% of the working volume of the tank. During the milling process, the tank was cooled continuously using an ice bath jacket. The Disruption process was performed on 5 consecutive cycles each consisting of three minutes of homogenizing and three minutes' break. One volume of buffer A was used to wash the glass beads and the total volume of homogenate was centrifuged at 29 000 g for 1 h. The supernatant was collected and dialyzed against buffer A.

Desalting

Solubilization and Purification

For performing the purification in one step we used a set of different columns in this process. firstly, the desalting step was performed by using a Bio-Gel P-6 column (Bio-Rad, USA). This provides an efficient and gentle gel filtration of sensitive compounds. Possible applications are desalting or buffer exchange due to size exclusion [17, 18]. According to the company instructions, the Gel P-6 column was washed four times using 20 mM potassium phosphate buffer, pH=8.0 centrifuged for 3 min at 1,000 x g. after that, 50 µl of the supernatant was pipetted to the Gel P-6 column. the column was centrifuged for four min at 1,000 x g. The sample was eluted using 500 µl of Tris buffer (10 mM Tris-HCl, pH 7.4) with 0.02% sodium azide.

Purification of ADH

The separation of ADH is performed by affinity chromatography using a Blue Sepharose column, which binds ADH effectively. After the collection of ADH on the column, the elution of the purified enzyme is implemented with NAD+[19].

The treated extract was injected into a Blue Sepharose column (1.5 · 13 cm) previously equilibrated by treatment in buffer A. Using 200mL of buffer the column was washed then the ADH enzyme was eluted with 0 to 2 mM NADP in buffer A.

The activity peak was collected and concentrated before being applied onto a Superdex 200-HR (1 · 30 cm) connected to a Waters HPLC system. Chromatography was performed in 50 mM NaH2PO4 pH 7, 0.15 M NaCl, 0.5 mM dithiothreitol and 20% glycerol at 0.4 mLÆmin)1. The pure protein was stored at 20 C in this buffer.

Electrophoretic analysis

The SDS/PAGE protein denaturation was executed as described [20, 21] using a proportion of 12% acrylamide. Proteins were

stained with silver nitrate. Native gel electrophoresis in 6% acrylamide was performed in Tris/boric/EDTA buffer, pH 8. Gels were incubated for 15min on ice in 20mM BisTris, pH 7, containing 1 mM NADPH for activity staining. A filter paper, soaked in 10 mM pentanal, 20 mM BisTris, pH 7, was placed covering the gels. After 5 min the filter paper was removed and the gel exposed to UV light. The elimination of fluorescence produced by NADPH indicated the activity of aldehyde reduction [7].

Enzyme activity

Kinetic parameters were determined at 25 °C CinaCary400 spectrophotometer (Varian, USA). The reduction of aldehydes was assayed in a 1 mL reaction mixture containing33 mM NaH2 PO4, pH7.0, 0.2mM NADPH, and 1 mM aldehyde by measuring the decrease of absorption at 340 nm. Signal was recorded at 365 nm with cinnamaldehyde, veratraldehyde, and anisaldehyde and at 400 nm for coniferaldehyde, using previously reported molar extinction coefficients **[3] [22]**. The oxidation of alcohol was performed in 0.1 M glycine, pH 10.0, 1.2 mM NADP and 10 mM of each alcohol, by measuring the rate of reduction of NADP at 340 nm. A wavelength of 365 nm was used for cinnamyl alcohol and of 400 nm for coniferyl alcohol oxidizing activities (e400¼ 27.5 mM)1Ækm)1 at pH 10.0). One unit of activity corresponds to 1 mol of NADP(H) formed per min.

RESULTS AND DISCUSSION

Elucidation of SDS-PAGE

Several bands are visible for the ADH standard since the tertiary and secondary structures of ADH are destroyed by SDS and DTT; the bands of the ladder are labeled with the belonging molar masses **(Tab. 1, Fig. 1)**. The rational explanation of the several bands is attributed to the presence of ADH subunits, the possibility of ADH degradation and impurities such as keratin **[7, 19]**. The most intense band at about 40 kDa was used as a reference for the samples. The aliquots A1 and A2 are overloading the SDS-PAGE and accordingly they cannot be precisely evaluated. However, two bands matching the ADH standard could be seen in the 40 kDa region which points to the presence of ADH enzyme but unlikely in a non-purified form. The protein content in A1 and A2 is very high and further purification is needed. However, improvement can already be seen between A1 and A2. The ADH band of A3 is clearly visible in Figure 1, it was clearly observable at the SDS-PAGE itself. The logarithm of the ladder masses is plotted against the calculated RF values to receive a regression curve using a third-degree

polynomial function as an approximation **(Fig.2)**.

Bradford determination

The quadratic function gives the best result for the determination coefficient including all standards and the origin;Fig.3shows the Bradford calibration curve. **Tab. 2** illustrates the calculated concentration depending on the absorbance using the Bradford determination.

The equation of the regression curve can be used for the calculation of the total protein concentration using the measured absorbance of the aliquots.

Enzyme activity

The ADH catalyzes the oxidation of ethanol to acetaldehyde by the simultaneous reduction of NAD^+ to NADH. In order to prevent the reverse reaction, the acetaldehyde reacts with semicarbazide to ethanone semicarbazone. The reaction equation is given in **Fig. 4**.

The amount of formed NADH is proportional to the consumption of the amount of ethanol, which also depends on ADH. NADH can be measured easily by UV absorption at 340 nm and thus indicates the enzyme activity. The UV instrument measured the absorption as a function of the time and automatically calculated the linear slope. For the calculation of the enzyme activity depending on volume v, the slope m of the samples was divided by the extinction coefficient ε and multiplied with the dilution factor f.

There are two possibilities for the declaration of the enzyme activity:

1. **Unit U (1 U = µmol/min)**
2. **Katal kat (1 kat = mol/s)**

Although the unit is the old enzyme unit, the handling of the values in this unit is easier and will be used in the calculation.

The specific activity s is calculated by the enzyme activity depending on volume V divided by the total protein concentration β **(Tab. 3, Fig. 5)**.

The respective variations in enzyme activity, the total protein concentration and the specific enzyme activity by means of the purification steps are illustrated in separate graphs **(Tab. 3, Fig. 6)**.

The enzyme activity decreases during the preparation steps as a proportional decrease in the total protein concentration **(Tab.3, Fig. 5)**. The aim of the purification was to minimize all impurities including all proteins except for ADH. This means that all proteins including all enzymes are counted for the total protein concentration. These enzymes possess an enzyme activity. When the total protein concentration decreases, fewer enzymes can generate an enzyme activity

[23]. Thus, a decrease in enzyme activity is observed. In order to make a statement of the quality of the purification steps, the specific enzyme activity is important. The specific enzyme activity shows the activity of a specific enzyme, in this case, ADH, per mass of protein. As depicted in **(Tab.3, Fig.6),** the purification step "desalting" between aliquot 1 and 2 increased the specific enzyme activity. Impurities such as small molecules, proteins, and salts, which are not from interest, are removed by size exclusion chromatography

This can be confirmed by a decrease in the total protein concentration as well as the reduction of the enzyme activity. In the following purification step, the same effect can be detected. During the affinity chromatography, additional proteins including enzymes were removed. However, the specific enzyme activity shows another result than expected. Instead of a significant increase due to the purification, the specific enzyme activity almost did not change. Since the total protein concentration and the enzyme activity decreased, while the specific enzyme activity stayed almost constant, the ADH is also removed during this purification step. The hypothesis is made that the affinity chromatography did not work properly, which can have multiple reasons such as overloading of the column, the method itself, the column itself, the pH value, impurities in the system or on the column and others **[24-26]**. The real reason could not be specified. The comparison of the recorded affinity chromatogram **(Fig.7)** with a reference affinity chromatogram **(Fig.8)** shows an unknown peak at the beginning of the measurement, which can be caused by the above-mentioned reasons. The broad small peak at 12-19 mL results from the remaining salts of the desalting step confirmed by the conductivity.

With these results, the SDS-PAGE is reconsidered. The decrease in the protein concentration is confirmed by the change of the color intensity of the stained proteins in the aliquots A1-A3. However, the quality of the purification steps cannot be determined due to the overload of the SDS-PAGE. By means of the photometric measurement and the resulting calculation of the specific enzyme activity, it can be clarified, that the desalting was successful for the purification since the specific enzyme activity increased from 0.42 U/mg to 0.947 U/mg, which is an increase of 125 %$p< 0.05$.

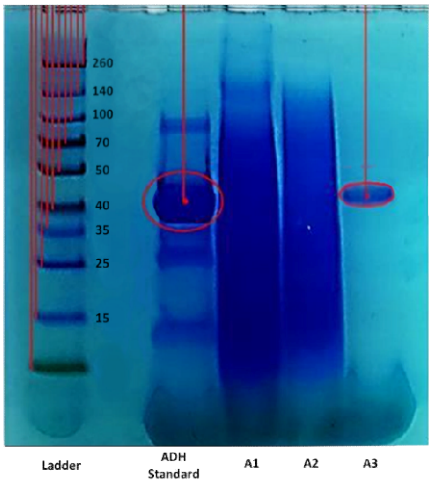

Figure 1: Shows the SDS-PAGE including the protein ladder, the ADH standard, and the three different samples A1-A3. The samples were taken after the cell disruption (A1), after the desalting (A2) and after the affinity chromatography (A3)

Table 1: Shows the SDS-PAGE including the protein ladder, the ADH standard, and the three different samples A1-A3. The samples were taken after the cell disruption (A1), after the desalting (A2) and after the affinity chromatography (A3)

Substance	distance starting line to analyte [cm]	Distance starting line to front [cm]	RF value	Molecular mass M [kDa]	log (M)
ladder 1	1.57	12.14	0.13	260	2.41
ladder 2	2.36	12.14	0.19	140	2.15
ladder 3	3.13	12.14	0.26	100	2
ladder 4	3.76	12.14	0.31	70	1.85
ladder 5	4.51	12.14	0.37	50	1.7
ladder 6	5.55	12.14	0.46	40	1.6
ladder 7	6.09	12.14	0.5	35	1.54
ladder 8	7.14	12.14	0.59	25	1.59
ladder 9	8.57	12.14	0.71	15	1.45
ladder 10	10	12.14	0.82	10	1.32
ADH standard	5.32	12.14	0.44	43.53	1.64
A3	5.14	12.14	0.48	39.88	1.6

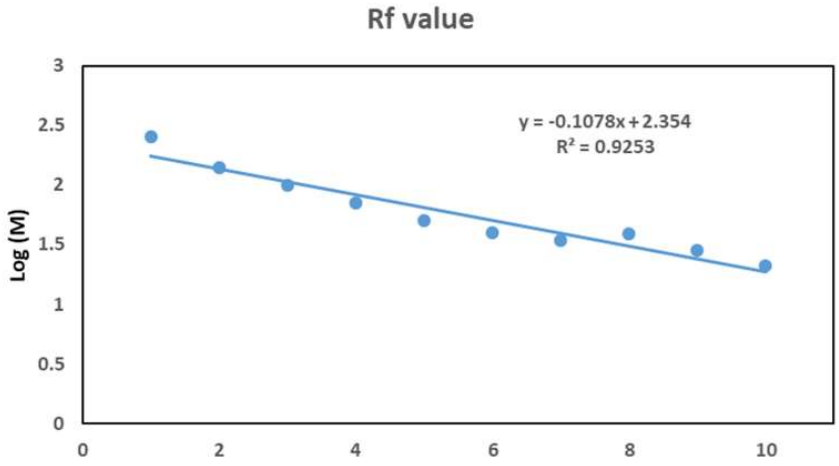

Figure 2: The relative mobility of the ADH enzyme

Table 2: calculated concentration depending on the absorbance using the Bradford determination

Aliquot	Absorbance	βdiluted sample [mg/mL]	Dilution Factor	βsample [mg/mL]
A1	0.324	0.319	33.33	10.63
A2	0.147	0.137	14.29	1.95
A3	0.029	0.026	5	0.13

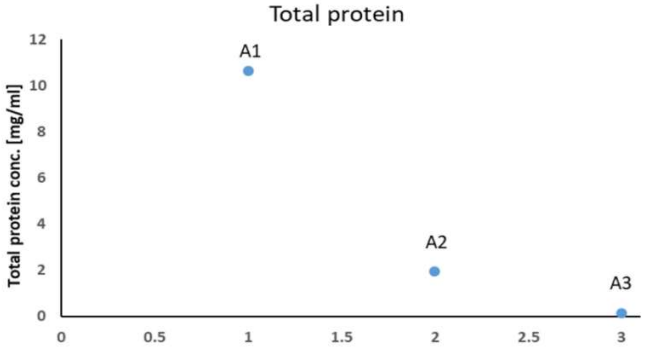

Figure 3: Illustration of the total protein concentration between the purification steps Bradford determination of total protein concentration

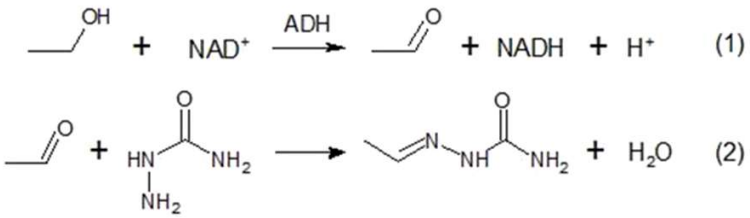

Figure 4: Reaction equations (1) oxidation of ethanol to acetaldehyde (2) further reaction of ethanol with semicarbazide to ethanone semicarbazone

Table 3: The specific activity

Aliquot	Slope[A/min]	Volume [µL]	Dilution Factor	Enzyme activity [U/mL]	Enzyme activity [kat/mL]	Specific enzyme [U/mg]	Specific Enzyme [kat/mg]
A1	0.693	50	40	4.47	7.45E-08	0.421	7.01E-09
A2	0.554	100	20.5	1.83	3.05E-08	0.931	1.51E-08
A3	0.036	100	20.5	0.12	1.98E-09	0.947	1.64E-08

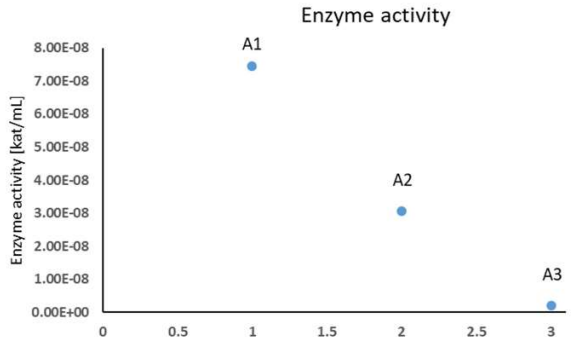

Figure 5: Illustration of the enzyme activity between the purification steps

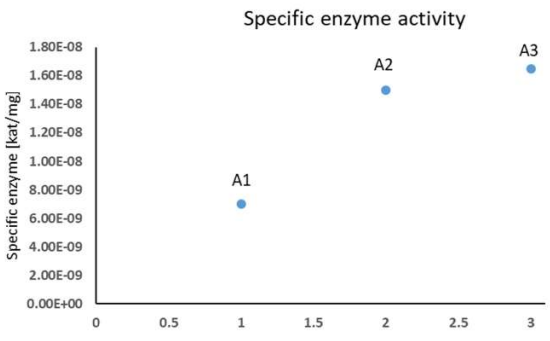

Figure 6: Illustration of the specific enzyme activity between the purification steps

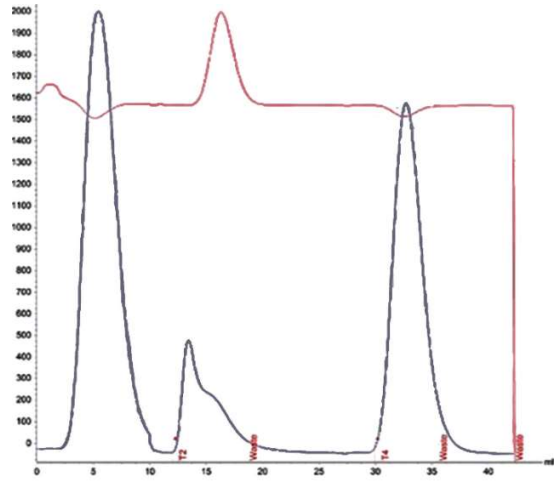

Figure 7: Recorded affinity chromatogram UV signal (blue line), conductivity (red line), Fraction (red text)

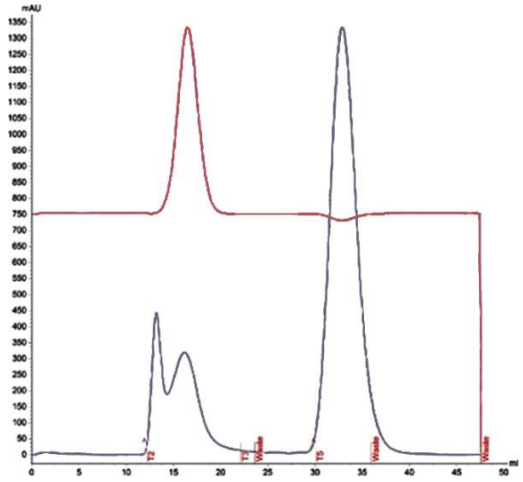

Figure 8 Reference affinity chromatogram UV signal (blue line), conductivity (red line), Fraction (red text)

CONCLUSION

The desalting treatment step is highly recommended to rule out unspecific proteins and enzymes concomitantly extracted during alcohol dehydrogenase purification process in *Saccharomyces cerevisiae*as well as it confers an escalation in both quality and quantity of the extract.

Conflict of interest

The authors declare no conflict of interest

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