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Short communication

Separation of manganese peroxidase isoenzymes on strong anion-exchange monolithic column using pH–salt gradient

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Abstract

Different chromatographic methods including chromatofocusing are used for separation of manganese peroxidase (MnP) isoforms and their isolation from the fungal growth medium. We tested strong anion exchange methacrylate based monolithic columns as a stationary phase for fast separation of MnP's. Sodium acetate buffers of two different pH values (6 and 4) were used for formation of reproducible pH gradient. The entire cycle, involving analysis and column regeneration, was completed in 3 min. Use of pH gradient showed better MnP isoform separation comparing to the salt gradient, while application of combined pH–salt gradient, resulted in further improvement.

Keywords: pH gradient; Salt gradient; Manganese peroxidase isoenzymes

1. Introduction

Excretion of manganese peroxidase (MnP) is widely distributed among fungi that intensively degrade lignin in woods [1,2]. Besides MnP, also lignin peroxidase (LiP) as well as laccase (Lac) are involved in lignin degradation. Both extracellular peroxidases are checked also due to their ability to degrade different xenobiotics [3,4]. They are families of structurally similar isoenzymes which are excreted into the growth medium depending on the composition of the media, the organism employed and the age of the culture [5,6]. The isoenzyme composition is important due to different catalytic properties of particular isoenzyme [7]. While for LiP's separation HPLC technique with an anion exchanging stationary phase using the concentration gradient of sodium acetate is mainly employed [8] for separation of MnP's different methods are proposed in literature. Diversity of methods, mobile phases and stationary phases [1,9] is required because of relatively high number of MnP isoforms which differ with the cultivation conditions as well as with the organism and strain employed. While the resolution of peaks in ion exchange mode can be low due to a similarity of MnP isoenzymes, the presence of LiP isoforms can additionally render their separation. Besides ion exchange chromatography chromatofocusing can be a method of choice [10,11].

Chromatofocusing is based on the formation of pH gradient inside a chromatographic column as the buffering species in the elution buffer titrate weak electrolyte functional groups on the stationary phase. Using this technique excellent separation in a very short time can be obtained [13]. Limitation of usage of a low buffer concentration required in a conventional cromatofocusing was overcome by introduction of gradient chromatofocusing [14]. Despite this improvement polymeric ampholyte buffers or complex low molecular mass buffers are still required for pH gradient formation in both cases.

Separation using pH gradient, is an alternative separation method, which also proved to be suitable for certain applications like separation of oligonucleotides [15] and antibodies [12] or their fragments [16]. Here strong ion-exchange resin, which is not involved in the pH gradient formation, is used.

For short bed monolithic columns, having a column length of down to 3 mm, commercially available under the trademark CIM Convective Interaction Media® [17] no formation of the pH gradient inside the column is expected. Due to a monolithic structure the mass transfer between the mobile and the stationary phase is significantly enhanced resulting in extremely fast separation of large molecules like proteins or

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Table 1
Stepwise concentration of the fungal growth medium containing MnP isoenzymes

Sample	MnP activity (U/l)	Volume (ml)	Protein concentration (mg/l)	Specific MnP activity (U/mg)
Crude growth medium	2280	250	239.4	9.5
Concentrated growth medium	20780	25	741.0	28
Fraction from DEAE Sepharose	34648	5	654.6	52.9

DNA [17]. Because of that, application of pH gradient over chromatofocusing is preferred, since shorter separation times are possible [16]. While all separations performed so far on CIM ion-exchange monolithic columns employed usage of salt gradient, we tested whether a fast separation of proteins using a pH gradient and a strong ion-exchange column is possible too. In this work we investigated reproducibility of pH gradient, time required for column equilibration and application of pH and pH–salt gradient for purification of MnP's produced by *Phanerochaete chrysosporium*.

2. Materials and methods

2.1. MnP production

P. chrysosporium MZKI B-223 (ATCC 24725) was grown in a nitrogen-limited medium [18] in agitated 500 ml Erlenmeyer flasks containing 100 ml of the growth medium at 32 °C. The growth medium contained 1 mM Mn(II) favoring MnP production only [19].

2.2. Growth medium pre-concentration

250 ml of *P. chrysosporium* growth medium was harvested after 4 days of cultivation, when the highest MnP activities were recorded. The growth medium filtrate was frozen overnight and rethawed to remove mucilaginous polysaccharides by centrifugation [20] but no changes in protein content and MnP activity occur. The growth medium was concentrated to the volume of 25 ml by ultrafiltration first and than dialyzed against 10 mM sodium acetate (pH 6). For both steps the membranes with cut off 10 kDa were used, therefore, the overall protein concentration and MnP activity after dialysis remained similar as after ultrafiltration. Finally, MnP purification on DEAE Sepharose was applied [21]. All 25 ml of the dialyzed concentrated medium was loaded to the DEAE Sepharose column previously equilibrated by potassium phosphate (20 mM, pH 6.5). The column was thoroughly washed with a potassium phosphate (20 mM, pH 6) as well as by a 20 mM sodium succinate (pH 5) solution. The bound MnP was eluted in 5 ml fraction by mobile phase step change to 1 M sodium acetate (pH 6) and desalted on a PD-10 column (Pharmacia, Uppsala, Sweden) afterwards. Data on sample volume, protein concentration, and MnP activity for consecutive purification steps are presented in Table 1.

2.3. HPLC analysis

MnP isoenzymes were isolated using CIM disk monolithic column containing 0.34 ml CIM QA (quaternary amine) disk (BIA Separations, Ljubljana, Slovenia). Experiments were performed on a gradient HPLC system built with two Pumps 64, an injection valve with a 100 μl stainless steel sample loop, a variable wavelength monitor with a 10 mm optical path set to 280 or 409 nm (depending on samples) and with a 10 μl volume flow-cell, connected by means of 0.25 mm i.d. PEEK capillary tubes. HPLC hardware/software (data acquisition and control station), from Knauer (Berlin, Germany) was used in all fast analytical separations. For pH measurement pH meter MA 5740 (Metrel, Horjul, Slovenia) was connected to the HPLC system. Isoenzymes were separated with a linear gradient of sodium acetate of different concentrations (from 10 mM to 1 M) and pH values (from 4 to 6), at a flow rate of 4 ml/min.

2.4. Isoelectrofocusing

Three main MnP fractions, labeled as MnP1, MnP2, and MnP4 were pooled, desalted, concentrated and analyzed by analytical isoelectric focusing [7]. Proteins were focused in the range of pH 3–6 and the protein bands were stained by Comassie brilliant blue staining.

MnP activity was determined by the oxidation of 2,6-dimethoxyphenol [22,23]. *Protein concentration* was measured according the method of Lowry [24].

3. Results and discussion

MnP isoforms have their isoelectric points between 3.5 and 5.1 depending on the organism, culture media composition and the age of population [5,6]. It was already shown that LIP isoenzymes can be very fast separated and isolated using CIM columns [25] therefore we applied them also in the study of MnP isoforms separation. Different mobile phases are used for MnP's separation, however 10 mM sodium acetate, pH 6 is usually used as a loading buffer. To form a pH gradient in the range, which corresponds to isoelectric points of *P. chrysosporium* MnP's (between 4.2 and 5.1), 10 mM sodium acetate, pH 4 was used as a buffer B. The reproducibility of gradient formation was tested by continuously measuring the pH value of the mobile phase outflow (Fig. 1). Five consecutive experiments gave almost identical pH gradient. Besides the good reproducibility it is

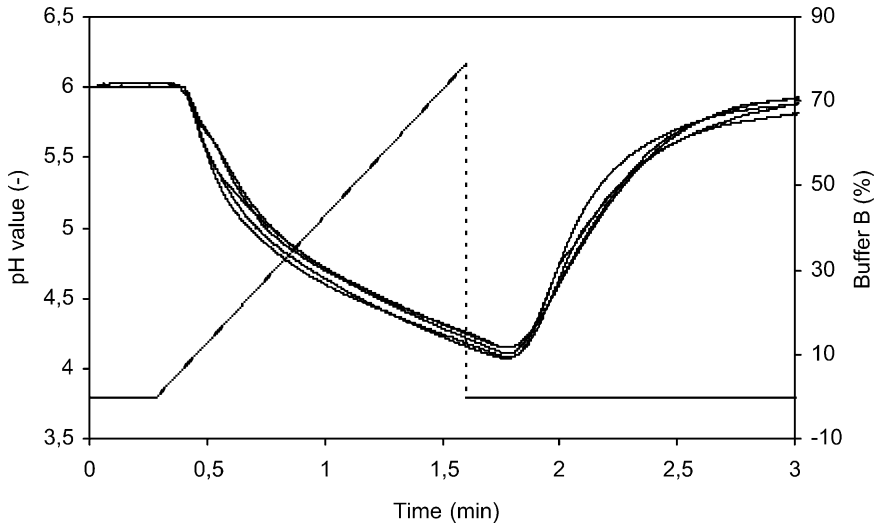

Fig. 1. Reproducibility of the pH gradient formation. Stationary phase: CIM QA disk monolithic column; mobile phase: buffer A: 10 mM sodium acetate, pH 6; buffer B: 10 mM sodium acetate, pH 4; gradient as shown in the figure; flow rate: 4 ml/min; detection: on-line pH-meter.

important that the initial pH value was completely restored in less then 1.5 min after the buffer A was pumped through the column again, enabling high frequency analysis without additional regeneration of the column. It is important to notice that entire cycle, comprising pH gradient and column re-equilibration, lasted only 3 min.

The reproducibility of the pH gradient enabled us to perform separation experiments with MnP sample. Three consecutive injections gave comparable chromatograms (Fig. 2) confirming that the method is robust enough for analysis of the sample isoenzyme composition. Furthermore, in comparison to widely used concentration gradient of sodium acetate [1,26] resolution obtained with pH gradient was significantly better (Fig. 2). Despite better resolution, separation was still not sufficiently good and no further improvement could be achieved changing slope of the pH gradient. Consequently, we tried to improve the resolution of peaks by combination of pH and salt concentration gradient. Concentration of buffer B was increased from 10 to 20 mM of sodium acetate, not changing the pH value (pH 4) what resulted in better separation of the isoforms (Fig. 3). Using the described method, the majority of non-hem proteins, strongly absorbing at 280 nm, was not retained at the support, while MnP isoenzymes, absorbing at 409 nm, were separated according to their decreasing p*I* value. Separation of all isoforms took place in the range, where pH value decreased almost linearly with time. All collected fractions exhibited MnP activity as tested by 2,6-dimethoxyphenol oxidation. The separation lasted less then 2 min while an additional minute is required for regeneration. As already observed from the chromatogram, IEF confirmed that beside major three MnP peaks characterized in Table 2 there is at least one isoform with the p*I* between MnP2 and MnP4 and at least two with the p*I* lower than MnP4 (Fig. 4). While

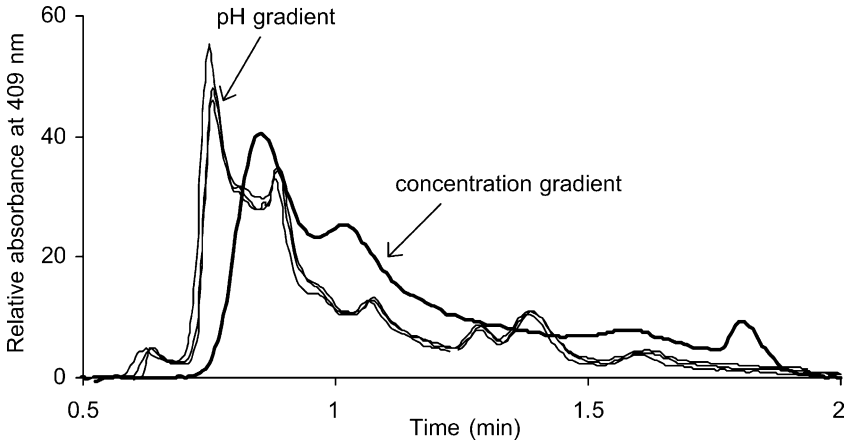

Fig. 2. Reproducibility of MnP isoenzyme separation using pH gradient and comparison with salt concentration gradient. Stationary phase: CIM QA disk monolithic column; mobile phase: pH gradient: buffer A: 10 mM sodium acetate, pH 6; buffer B: 10 mM sodium acetate, pH 4; concentration gradient: buffer A, 50 mM sodium acetate, pH 6; buffer B, 200 mM sodium acetate, pH 6; gradient: 0–100% of buffer B in 100 s; sample: 100 μl of desalted Sepharose eluat; flow rate: 4 ml/min; detection: Vis at 409 nm.

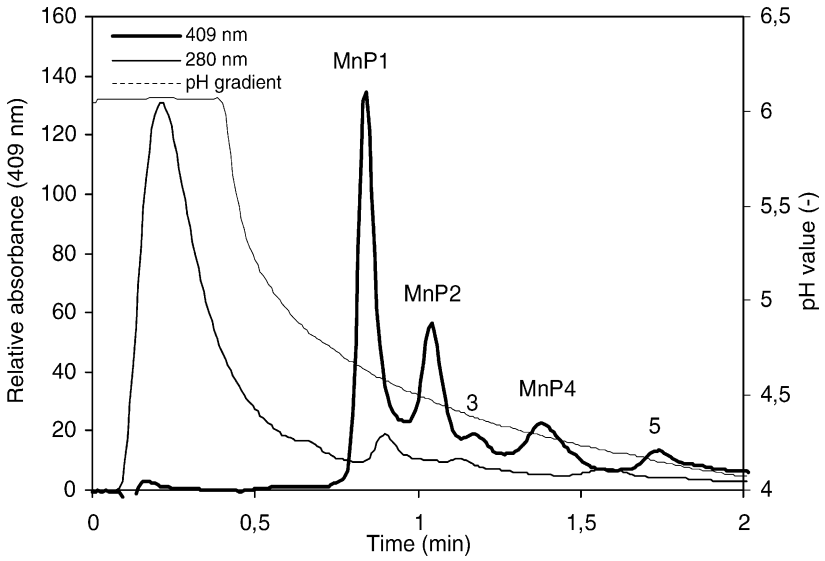

Fig. 3. Separation of MnPs by combination of pH and concentration gradient. Stationary phase: CIM QA disk monolithic column; mobile phase: buffer A: 10 mM sodium acetate, pH 6; buffer B: 20 mM sodium acetate, pH 4; gradient: 0–100% of buffer B in 100 s; sample: 100 μl of desalted Sepharose eluat; flow rate: 4 ml/min; detection: UV at 280 nm, Vis at 409 nm and on-line pH-meter.

Table 2
Specific activities and p*I* values of three isolated MnP isoenzymes

MnP isoenzyme	Specific activity (U/mg)	p*I* value
MnP1	59.1	4.9
MnP2	121.7	4.6
MnP4	123.1	4.2

MnP1 was isolated mainly as a homogenous MnP fraction, it was not possible to obtain MnP2 isoform without at least a trace of MnP1. There were also isoforms with lower p*I* values presented in the isolated MnP4 fraction. Nevertheless, significant improvement in purity was achieved in comparison to the composition of the entire fraction retained on CIM monolithic column (Fig. 4, column 2).

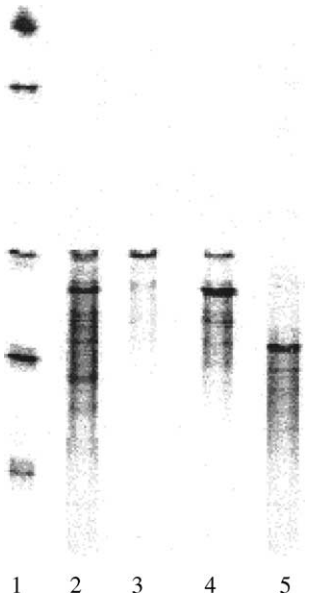

Fig. 4. IEF gel. Lanes: 1, IEF standard; 2, fraction retained on CIM monolithic column; 3, MnP1; 4, MnP2; 5, MnP4.

It can therefore be concluded that CIM ion-exchange monolithic columns can be efficiently applied for fast separation of proteins and also for their purification using, besides salt also pH gradient.

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