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An easy two-step purification method for human leucocyte myeloperoxidase

Abstract: Objective: The object of this study is to describe a simple, rapid and cost effective method for purification of human leucocyte myeloperoxidase from a single donor. Myeloperoxidase (MPO) was purified by a two step procedure consisting of concanavalin-A Sepharose 4B affinity chromatography followed by CM-Sephadex cation exchange chromatography.

Methods: Leucocytes from a single donor collected by leucopheresis were used in purification studies. MPO was solubilized and extracted from leucocytes by homogenization in phosphate buffer containing 1% HETAB (hexadecyltrimethylammonium bromide). MPO containing soluble material was applied onto concanavalin-A Sepharose 4B affinity gel, and was eluted with methyl-a-D-manno-piranoside. Fractions with MPO activity were pooled, dialyzed and applied onto CM-sephadex cation exchange gel, and was eluted from the column at weak cationic pH with linear NaCl gradient.

Results: By the use of two chromatographic procedures, MPO was purified from human leucocytes with 70% yield. Purity of MPO was checked by determining the Reinheit Zahl (RZ) value (\(A_{430}/A_{280}\)). The RZ value of 0.86 indicated that the purified enzyme was highly homogenous as compared to reported experimental values (ranging from 0.82 to 0.88) and pure commercial enzyme with the RZ value of 0.84.

Conclusion: In comparison with earlier purification methods, the purification method reported here has higher recovery rate and high purity together. Use of leucocytes with leucopheresis origin help us to omit the leucocyte isolation step and omitting of ammonium sulphate precipitation steps also help us to reduce the cost and is shortened the time of purification.

Keywords: Myeloperoxidase, human leucocytes, purification

Özet: Amaç: Bu çalışmanın amacı, tek vericiden temin edilen lökositlerden miyeloperoksidaz (MPO) enzimini saflaştırmak için basit, hızlı ve ekonomik bir yöntem geliştirmektir. Çalışmada, insan lökosit miyeloperoksidaz enziminin konkanavalin A-Separoz 4B afinite kromatografisi ile CM-Sepadeks katyon değiştirici kromatografisinden oluşan iki basamak saflaştırılması sunulmuştur.

Metod: Saflaştırma çalışmasında, lökoferez ile tek kişiden alınmış insan lökositleri kullanıldı. Lökosit miyeloperoksidazı, %1 oranında HETAB (hezkadesiltrimetilamonyum bromide) içeren fosfat tampon içinde çözünürleştirildi ve santrifügasyonla diğer zarsal fraksiyonlardan ayrılrıldı. Çözünürleştirilen enzim konkanavalin A-Separoz afinite jeline uygulandı ve bağlanan enzim bu jelden metil-a-D-manno-piranozid ile indirildi. Kromatografide MPO aktivitesi içeren fraksiyonlar birleştirildi, diyaliz edildikten sonra CM-sefadeks katyon değiştirici kolona uygulandı. Enzim bu kolondan hafif bazik pH’daki fosfat tampon içinde lineer NaCl gradient ile indirildi.

Bulgular: Uygulanan iki kromatografik yöntemble MPO enzimi insan lökositlerinden %70 verimle saflaştırıldı. Enzimin sağlığı, Reinheit Zahl (RZ) değeri (\(A_{430}/A_{280}\)) ölçülenler korelasyon kontrol edildi. Reinheit Zahl değeri saflaştırılan MPO için 0.86 olarak hesaplandı. Bu RZ değeri, daha önce literatürde safl enzim için bildirilen değerler (0.82–0.88)
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Introduction

Myeloperoxidase (E.C. 1.11.1.7. Donor: H₂O₂ oxidoreductase) is a heme-containing protein found in the primary granules of neutrophils and monocytes. It is an extremely abundant protein, comprising up to 5% of the total protein in neutrophils and up to 25% of granule proteins [1]. The main function of myeloperoxidase is to destroy phagocytosed microorganisms by generating highly reactive hypo-chlorous acid (HOCl) and hypothiocyanous acid (HOSCN) within the phagosome. The generation of these reactive species is dependent on the presence of H₂O₂ and a halide, such as chloride or cyanide [2,3].

Reactive species formed by myeloperoxidase are necessary for antibacterial activity of leucocytes. These species may also cause tissue damage through the modification of lipids and proteins. Important biological modifications resulting from myeloperoxidase activity include tyrosyl radical driven protein cross linking [4], reactive chloride mediated cholesterol oxidation [5], and hypochlorous acid dependent conversion of free amino acid to reactive, diffusible aldehydes [6]. Myeloperoxidase has been shown to be present and active in human atherosclerotic lesions [7], possibly contributing to the progression of the disease.

All these features of MPO make this enzyme an important molecule for researchers and preparing large amounts of enzyme is an important step for these studies. In previous studies, MPO has been purified from various sources including canine pus [8], human leukocytes [9,10], chinese hamster ovary cells transfected with recombinant human myeloperoxidase [11] and HL60 promyelocytic cells [12] by the use of different methods. Here in this study we report a simple, fast and cost effective purification method for the purification of MPO from human leucocytes.

Materials and Methods

Materials

Human leucocytes were obtained from Hacettepe University Hospitals, Apheresis Center, Ankara, Turkey. NaCl, KCl, MgCl₂, KH₂PO₄, K₂HPO₄, NaH₂PO₄, Na₂HPO₄, H₂O₂, NaOH, Concanavalin A-Sepharose 4B affinity gel, CM-Sephacryl C-50 cation exchange gel, methyl-a-D-mannoside, 3,3′,5,5′-tetramethylbenzidine (TMB), N,N-dimethylformamide, hexadecyltrimethylammonium bromide (HETAB), ethylenediaminotetraacetic acid (EDTA), Bradford reagent, bovine serum albumin fraction V, ammonium persulphate (APS), N,N,N′,N′-tetramethylethylene-diamine (TEMED) were obtained from Sigma Aldrich Co. St. Louis, MO, USA. Acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), coomassie brilliant blue R-250, bromphenol blue were obtained from Bio-Rad Laboratories, Richmond, CA, USA. Chromatography columns were obtained from Amersham Bioscience Uppsala, Sweden.

Methods

Leucocyte homogenization and MPO extraction

Human leucocytes collected by leucopheresis were suspended in 25 mM sodium phosphate buffer containing 0.5 mM EDTA (pH:7.4) at a ratio of 1 ml of buffer to 1 ml of cell pellet. The mixture was gently mixed and centrifuged at 2 500 xg for 15 minutes. The pellet (leucocytes) was suspended in 10 mM sodium phosphate buffer (pH: 7.0) containing 1 mM MgCl₂ and 3mM NaCl (buffer A), and homogenized using a dounce homogenizer on ice. The resulting homogenate was then centrifuged at 20 000 xg for 15 minutes to obtain leucocyte membrane fractions containing MPO. The pellet was collectred and resuspended in buffer A at a ratio of 10 ml buffer to 1 ml of pellet. HETAB was then added to a final concentration of 1%, and the mixture was stirred gently for 2 h at 4°C. The insoluble material was removed by centrifugation at 20 000 xg for 15 min. Supernatant was collected and the pellet was resuspended in the same buffer and centrifuged again. The first and second supernatants were pooled and dialyzed overnight at 4°C against buffer A.
Figure 1: Concanavalin A Sepharose 4B affinity chromatography. Peak A: proteins in void volume, Peak B: weakly bound proteins eluted with 1M NaCl, Peak C: MPO eluted by 0.6 M methyl α-D mannopyranoside in 1 M NaCl. (x - - - x): protein, (o - - - o): MPO activity.

Figure 2: CM-Sephadex cation exchange chromatography. Peak A: proteins in void volume, Peak B: high MPO activity peak eluted with NaCl gradient, Peak C: proteins eluted with high NaCl. (x - - - x): protein, (o - - - o): MPO activity, (Δ.....Δ): NaCl gradient.
Concanavalin-A Sepharose 4B chromatography

All chromatography studies were carried out at 4°C. Dialyzed material was applied onto concanavalin-A Sepharose 4B column (2.6 x 6 cm) equilibrated with 100 mM sodium acetate buffer (pH 6.3) containing 100 mM NaCl, 1 mM CaCl₂, 1mM MgCl₂, 0.5 mM EDTA and 0.1% HETAB (buffer B), at a flow rate of 0.6 ml/min. After application of the sample, the column was washed with equilibration buffer until $A_{280}$ returned to baseline. Then the column was rewashed with the same buffer containing 1M NaCl to remove weakly bound proteins to the gel. MPO was then eluted from the column with 600 mM methyl-a-D-manno-piranoside in the buffer B containing 1 M NaCl. Fractions were assayed for MPO activity and protein content. MPO containing fractions were pooled, dialyzed against buffer B to remove NaCl and methyl-a-D-manno-piranoside, and used in ion exchange chromatography.

Ion exchange chromatography

After overnight dialysis against buffer B, the sample was applied to CM-Sephadex cation exchange column equilibrated with buffer B (85 ml gel in a 2.6 x 16 cm column) at a flow rate of 0.6 ml/min. After washing with two bed volumes, the column was washed with the same buffer of pH 8.0. The absorbance of the wash at 280 nm was checked and the enzyme was eluted from the column with a linear gradient of NaCl (0-1M) in 100 mM sodium acetate buffer containing 100mM NaCl, 1mM CaCl₂, 1mM MgCl₂, 0.5mM EDTA, and 0.1% HETAB (pH:8.0). Fractions with high MPO activity were pooled, concentrated and analyzed for purity.

MPO activity assay

The activity of MPO was measured by the method of Suzuki et al. modified by Demirpence et al. [13,14]. MPO activity was measured in a final volume of 1 ml containing 80 mM phosphate buffer (pH: 5.4), 0.5% HETAB, 1.6 mM synthetic substrate tetramethylbenzidine (TMB) initially dissolved in dimethylformamide, and 2 mM H₂O₂. The reaction was started at 37°C by the addition of H₂O₂. The initial rate of MPO-catalyzed TMB oxidation was followed by recording the increase of absorbance at 655 nm. One MPO unit was defined as the amount of the enzyme producing one absorbance change per minute under assay conditions.

Protein assay

During purification studies including column chromatographies, the protein concentration was determined by measuring the absorbance of samples and fractions in 280 nm. Protein concentration of purified enzyme was measured by BCA method [15,16].

Results

Since myeloperoxidase is found in the particulate fraction of leucocytes (primarily neutrophils), removal of soluble
proteins and solubilization membrane associated enzyme using a suitable detergent (HETAB or CETAB) is an important step in the purification of the enzyme. The cytosolic and particulate fractions were separated by homogenization followed by centrifugation, and the particulate fraction was solubilized with 1% HETAB.

In Concanavalin-A Sepharose 4B chromatography, non glycosylated proteins were recovered in void volume, during sample application and washing the column with Buffer B. This protein fraction contained some peroxidase activity arising from other hemoproteins. Peroxidase activity of this fraction was not considered as MPO and was discarded (Figure 1, peak A).

Weakly and non-specifically proteins that bound to Con A-Sepharose were removed in the next washing step with Buffer B containing 1M NaCl (Figure 1, peak B). MPO was then eluted from the column with Buffer B containing 1 M NaCl plus 600 mM methyl-a-D-manno-piranoside (Figure 1, peak C). This protein peak contained MPO, and other proteins that were removed in the next step, in cation exchange chromatography.

Fractions with MPO activity from Con A Sepharose chromatography were pooled, dialysed against Buffer B of pH 6.3, and applied to cation exchange (CM-Sephadex resin pre equilibrated with the same buffer. After sample application, the column was washed with Buffer B of pH 8.0. No MPO activity was detected in the eluate neither during sample application nor washing. These fractions were discarded after activity check. After extensive washing, cation-exchange resin bound MPO was eluted using a linear gradient of NaCl in Buffer B of pH 8.0. With NaCl gradient MPO was eluted as a sharp activity peak followed by an activity shoulder (Figure 2, peak B and C). Fractions with high MPO activity and low protein (Figure 2, peak B) were pooled and concentrated.

The purity of purified MPO was determined by SDS-PAGE (Figure 3) and measuring the Reinheit Zahl (RZ) value ($A_{430}/A_{280}$). The ratio between the absorption at the Soret peak and that at 280 nm (the RZ value) is commonly used as a criterion for purity of hem peroxidases. The final myeloperoxidase preparation had an RZ value of 0.864 in our study (Figure 4). The RZ values reported ranges from 0.82 to 0.88 for pure enzyme preparations in previous studies [12,17,18]. Both electrophoresis and RZ value showed that the purified enzyme is highly homogenous.

The recovery of human leukocyte myeloperoxidase activity at each stage of purification is given in Table 1. When leukocyte membrane homogenate was considered as starting material, the purification fold was 13 with a final yield was 70.3% and the purified enzyme had a specific activity of 110.7 U/mg protein.

**Discussion**

The lectin Con A binds molecules containing a-D-mannopyranosyl, a-D-glucopyranosyl and sterically related residues. Therefore this protein can be used for applications such as separation and purification of glycoproteins including myeloperoxidase.

MPO was first isolated by Kjell Agner from emphysema fluid of tuberculosis patients [19] using a 5 step multiple precipitation method. Later he reported the purification of MPO from pus of infected dog uteri and human blood with a long 6 step precipitation method followed by adsorption to kiseslguhr [20] or silica gel chromatography [21].

Several studies have been published for the isolation of myeloperoxidase from human blood and from other sources. Bakkenist et. al demonstrate a 3 step method

**Table 1:** Purification process for human leucocyte myeloperoxidase.

| Purification step                             | Total protein (mg) | Total activity (Units) | Specific activity (Units/mg) | Purification (Fold) | Yield (%) |
|----------------------------------------------|-------------------|-----------------------|-------------------------------|---------------------|-----------|
| Leucocyte membrane homogenate                | 11751.8           | 103950.0              | 8.8                           | 1.0                 | 100.0     |
| Concanavalin-A Sepharose chromatography      | 1010.5            | 83190.0               | 82.3                          | 9.7                 | 80.0      |
| CM-Sephadex cation exchange chromatography   | 660.8             | 73125.0               | 110.7                         | 13.0                | 70.3      |
with one precipitation and two nonspecific chromatographic steps. Despite of simplicity of method, lose of 83% of activity is a great disadvantage [20]. Similar procedure with little changes applied by Matheson et al. with recovery yielded of 79% and purity of 76% [10].

Concanavalin A-sepharose first was used in the purification of horseradish peroxidase [21] and rat mammary tumor peroxidase [22]. Con A-sepharose was used in purification of human leucocyte MPO by Douglas P. Merrill [23] and Olsen and Little [9]. Merrill demonstrated a two step method starting with concanavalin A-sepharose affinity chromatography flowed by gel filtration. He reported a recovery yield and final purity more than 100% that made his report unacceptable.

Olsen and Little reported a 3 step chromatographic method for the purification of MPO. They applied affinity chromatography, hydrophobic interaction and gel filtration chromatography and reported near 100% purity and 40% yield of recovery. Concanavalin was also used for isolation of MPO from HL60 cell lines [12]. Myeloperoxidase was purified from other sources such as equine blood [24] and fish neutrophils [25] with similar results. Most of methods had high purity rate because of high abundance of enzyme but recovery rate is low.

One mg of pure crystalline MPO has an absorption value of 1.2 at 430 nm. Since the first purification of MPO, the ratio of $A_{430}/A_{280}$ is considered as a marker for the purity of the enzyme. This ratio, known as the Reinheit Zahl (RZ) value is reported from 0.82 to 0.88 in the so far published MPO studies [20].

In comparison with earlier purification methods, the purification method reported here has higher recovery rate and high purity together. Low cationic pH in cation exchange chromatography instead of anionic pH involve to elution of weak cationic proteins in loading step and gives a better purification profile in cation exchange chromatography. The recovery rate of 70% and RZ value of 0.86 showed the success of our method. Use of leucocytes with leucopheresis origin help us to omit the leucocyte isolation step and omitting of ammonium sulphate precipitation steps also help us to reduce the cost and is shortened the time of purification.

Conflict of Interest: The authors have no conflict of interest.

References

[1] Segal AW. How neutrophils kill microbes. Annu Rev Immunol 2005;23:197–223.

[2] Wang JG, Mahmud SA, Thompson JA, Geng JG, Key NS, Slungaard A. The principal eosinophil peroxidase product, HOSCN, is a uniquely potent phagocyte oxidant inducer of endothelial cell tissue factor activity: a potential mechanism for thrombosis in eosinophil inflammatory states. Blood 2006; 107:558–65.

[3] Obinger C. Chemistry and biology of human peroxidases. Arch Biochem Biophys 2006; 445(2):197–8.

[4] Heinecke JW, Li W, Francis GA, Goldstein JA. Tyrosyl radical generated by myeloperoxidase catalyzes the oxidative cross-linking of proteins. J Clin Invest 1993; 91(6):2866–72.

[5] Hazen SL, Hsu FF, Duffin K, Heinecke JW. Molecular chlorine generated by the myeloperoxidase-hydrogen peroxide-chloride system of phagocytes converts low density lipoprotein cholesterol into a family of chlorinated sterols. J Biol Chem 1996; 271(38):23080–8.

[6] Hazen SL, Hsu FF, Heinecke JW. p-Hydroxyphenylacetalddehyde is the major product of L-tyrosine oxidation by activated human phagocytes. A chloride-dependent mechanism for the conversion of free amino acids into reactive aldehydes by myeloperoxidase. J Biol Chem 1996; 271(4):1861–7.

[7] Daugherty A, Dunn JL, Rateri DL, Heinecke JW. p-Hydroxyphenylacetalddehyde and its metabolites, p-hydroxyphenylacetaldehyde and p-hydroxyphenylacetone, are produced by human monocytes and neutrophils in the presence of H2O2 and chloride. J Biol Chem 1997; 272(24):15359–65.

[8] Harrison JE, Pabalan S, Schultz J. The subunit structure of crystalline canine myeloperoxidase. Biochim Biophys Acta 1977; 493(2):247–59.

[9] Olsen RL, Little C. Purification and some properties of myeloperoxidase and eosinophil peroxidase from human blood. Biochim J 1983; 209(3):781–7.

[10] Matheson NR, Wong PS, Travis J. Isolation and properties of human neutrophil myeloperoxidase. Biochemistry 1981; 20(2):325–30.

[11] Moguilevsky N, Garcia-Quintana L, Jacquet A, Tournay C, Fabry L, Piérard L, et al. Structural and biological properties of human recombinant myeloperoxidase produced by Chinese hamster ovary cell lines. Eur J Biochem 1991; 197(3):605–14.

[12] Hope HR, Remsen EE, Lewis C Jr, Heuvelman DM, Walker MC, Jennings M, et al. Large-scale purification of myeloperoxidase from HL60 promyelocytic cells: characterization and comparison to human neutrophil myeloperoxidase. Protein Expr Purif 2000; 18(3):269–76.

[13] Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leucocytes. Anal Biochem 1983; 132(2):345–52.

[14] Demircan E, Köksoy C, Kuzu A, Kilinc K. A spectrophotometric assay for tissue-associated myeloperoxidase activity and its application to intestinal ischemia-reperfusion. Turk. J. Med. Sci 1997; 27:197–200.

[15] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bichinchoninic acid. Anal Biochem 1985; 150(1):76–85.

[16] Stoscheck CM. Quantitation of protein. Methods Enzymol 1990; 182:50–68.

[17] Svensson BE, Domeij K, Lindvall S, Rydell G. Peroxidase and peroxidase-oxidase activities of isolated human myeloperoxidases. Biochem J 1987; 242(3):673–80.

[18] p. n. products. (2012). General Product Information. Available: http://www.myeloperoxidase.com/MPO/framedef.html.
[19] Agner K. Verdoxidase; a Ferment Isolated from Leucocytes. Acta physiol. Scand 1941.

[20] Bakkenist AR, Wever R, Vulsma T, Plat H, van Gelder BF. Isolation procedure and some properties of myeloperoxidase from human leucocytes. Biochim Biophys Acta 1978; 524(1):45–54.

[21] Brattain MG, Marks ME, Pretlow TG 2nd. The purification of horseradish peroxidase by affinity chromatography on Sepharose--bound concanavalin A1,2. Anal Biochem 1976; 72:346–52.

[22] DeSombre ER, Lyttle CR. Isolation and purification of rat mammary tumor peroxidase. Cancer Res 1978; 38(11 Pt 2):4086–90.

[23] Merrill DP. Purification of human myeloperoxidase by Concanavalin A-Sepharose affinity chromatography. Prep Biochem 1980; 10(2):133–50.

[24] de la Rebière de Pouyade G, Serteyn D, Deby-Dupont G, Franck T. Method for co-purification of equine neutrophil elastase and myeloperoxidase from a limited blood volume. Res Vet Sci 2009; 87(3):358–63.

[25] Castro R, Plazzon MC, Noya M, Leiro JM, Lamas J. Isolation and molecular cloning of a fish myeloperoxidase. Mol Immunol 2008; 45(2):428–37.