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Purification of Peroxidase Enzyme from Radish Species in Fast and High Yield with Affinity Chromatography Technique

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Abstract

In this study, an effective single step affinity method is presented for purifying plant peroxidase (POD) enzymes from radish species. This method make possible to purify the enzymes in high yield and purity. Briefly, 10 different 4-amino benzohydrazide derivatives were synthesized and identified as new competitive POD inhibitors. Then, these derivatives were coupled to Sepharose 4B-L-Tyrosine support matrix by diazotization to form the affinity gels. Purification factors were recorded as 54.8% yield - 665-fold, 33.8% yield - 613-fold, 22.7% yield - 595-fold, 34.4% yield - 781-fold, 40.9% yield - 282-fold for turnip (T-POD), black radish (BR-POD), daikon (D-POD), sweet radish (SR-POD) and kohlrabi radish, (KR-POD), respectively. It has also been shown that the affinity gels, which prepared using the 4-amino 3-bromo benzohydrazide and 4-amino 2-nitro benzohydrazide molecules, capable to purify all radish species POD enzymes in high purity and yield.

Keywords: Peroxidase, Purification, Radish, Affinity chromatography

1. Introduction

Peroxidases (POD, EC 1.11.1.7) are haeme containing enzyme groups that widely found in bacteria, fungi, plants and animals. Peroxidases are divided into 3 classes according to amino acid sequence similarities and metal-ion binding capacities (class I, prokaryotic peroxidases; class II, extracellular fungal peroxidases; and class III, plant secretory peroxidases) [1]. They have important roles in physiological processes in plants, such as protecting cells against oxidative stress, lignin and suberin formation, crosslinking of cell wall components, healing of wound, defense against pathogens or insects [2-5]. PODs have generally been used in clinical immunoassays, biochemical detection of glucose, uric acid and cholesterol, treatment of phenolic contaminations in wastewater and paper industries [6,7]. POD was firstly characterized from horseradish roots [8], then, different PODs were isolated, purified and characterized from many plant sources including turnip, radish, soybean, potato, beetroot, vanilla bean, apple and orange seeds, royal palm, tomato, date palm, black gram, olives, strawberries and bitter gourd [9-12]. For many years, horseradish plant has been used as the only commercial source for the production of plant PODs; however, POD can also be obtained from other radish species. The different PODs can be as efficient as HRP in terms of stability, yield and substrate specificity [13]. Therefore, improvements of existing procedures or developing new methods are commercially important.

There are many methods for purification of the PODs in literature. Ammonium sulfate precipitation [14], ion exchange chromatography [14, 15], affinity chromatography [16] and two-phase extraction coupled with gel filtration chromatography [17] are some of them. During the purification of PODs have been encountered with problems such as the multiplicity of the chromatographic methods, the increase of the cost, the low yield, and the poorly obtained enzyme when traditional methods are used. Purification of biomacromolecules including enzymes by affinity chromatography provides advantages over other chromatographic methods such as yield, purity and efficacy. Reversible inhibitors of peroxidase (RIP) are important molecules for affinity chromatography. RIP's can bind the POD and they contribute separation of the PODs. Some molecules including hydrazines and azides are known to be potent peroxidases inhibitors (PODIs) [18, 19]. These molecules were used for purification of PODs from turnip and radish [20].

In this study, new RIP's (10 different 4-aminobenzohidrazid derivatives) were determined and Sepharose-4B-L-Tyrosine affinity gels synthesized using the RIP molecules. According

to our results, use of the new RIP molecules allowed to single step purification of PODs from black radish, daikon, sweet radish, kohlrabi radish and turnip in high yield and purity.

2. Material and Methods

2.1. Material

Fresh radish spices were purchased from local shops. Methyl 4-aminobenzoate derivatives obtained from fluorochem ltd U.K., CNBr-activated-Sepharose-4B and L-tyrosine were purchased from Sigma–Aldrich Co. Prestained Protein Ladder (Thermo Scientific 26616) and all other chemicals used are analytical grade purchased from sigma Aldrich or Merck.

2.2. Synthesis of 4-amino benzohydrazide derivative compounds

4-amino benzohydrazide derivates (1a-10a) were synthesized from methyl 4-amino benzoate molecules (1-10). Synthesis mechanisms were presented in Figure 1. 3 mmol of 4-amino benzoate molecules (1-10) and 2 ml of 64% hydrazine monohydrate were added to 30 ml of absolute ethanol, and then the mixture was refluxed for 24 hours. After evaporation, the residue was added to ethanol and was heated-cooled to obtain crude crystals. The crude crystals were recrystallized from ethanol **[21]**. Synthesized molecules confirmed by 400 MHz ^{1}H-NMR (Bruker) and Q-TOF LC/MS (Agilent 6530 Accurate Mass).

4-Amino 2-methyl benzohydrazide (**1a**) - ^{1}H-NMR (400 MHz, DMSO-d_6): 9.00 (bs, 1H), 7.04 (d, *J* = 8.2 Hz, 1H), 6.34 (d, *J* = 2.1 Hz, 1H) 6.31 (dd, *J* = 8.2 Hz, *J* = 2.1 Hz, 1H), 5.29 (s, 2H), 4.25 (bs, 2H), 2.22 (s, 3H). HRMS (TOF MS ES+): m/z calcd for $C_8H_{12}N_3O^+$ $[M+H]^+$ 166.0975, found 166.0971.

4-Amino 2-methoxy benzohydrazide (2a) - ^{1}H-NMR (400 MHz, DMSO-d_6): 8.72 (bs, 1H), 7.57(d, *J* = 8.4Hz, 1H), 6.20 (d, *J* = 1.9 Hz, 1H) 6.17 (dd, *J* = 8.4 Hz, *J* = 1.9 Hz, 1H), 5.68 (bs, 2H), 4.37 (bs, 2H), 3.78 (s, 3H). HRMS (TOF MS ES+): m/z calcd for $C_8H_{11}N_3O_2^+$ $[M+H]^+$ 182.0924, found 182.0924.

4-Amino 2-nitro benzohydrazide (3a) - ^{1}H-NMR (400 MHz, DMSO-d_6): 9.47 (bs, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 6.95 (d, *J* = 2.2 Hz, 1H), 6.72 (dd, *J* = 8.4, 2.2 Hz, 1H), 6.07 (bs, 2H), 4.32 (bs, 2H). HRMS (TOF MS ES+): m/z calcd for $C_7H_9N_4O_3^+$ $[M+H]^+$ 197.0669, found 197.0672.

4-Amino 2-chloro benzohydrazide (4a) - ^{1}H-NMR (400 MHz, DMSO-d_6): 9.19 (t, J=4 Hz, 1H), 7.08 (d, J=8.4 Hz, 1H), 6.57 (d, J=2.4 Hz, 1H), 6.46 (dd, J=8.4 Hz, J=2.4 Hz, 1H); 5.69 (s, 2H); 4.34 (d, J=4 Hz, 2H). HRMS (TOF MS ES+): m/z calcd for $C_7H_9ClN_3O^+$ $[M+H]^+$ 186.0429, found 186.0427.

4-Amino 2-bromo benzohydrazide (5a) - ^{1}H-NMR (400 MHz, DMSO-d_6): 9.20 (s, 1H); 7.04 (d, J =8 Hz, 1H); 6.77 (d, J =2 Hz, 1H); 6.50 (dd, J=8 Hz, J=2 Hz, 1H); 5.65 (s, 2H); 4.33 (s, 2H). HRMS (TOF MS ES+): m/z calcd for $C_7H_9BrN_3O^+$ $[M+H]^+$ 229.9924, found 229.9926.

4-Amino 3-methyl benzohydrazide (6a)- ^{1}H-NMR (400 MHz, DMSO): 9.26 (bs, 1H), 7.47(s, 1H), 7.42 (d, *J* = 8.3 Hz, 1H), 6.56 (d, *J* = 8.3 Hz, 1H), 5.36 (s, 2H), 4.29 (s, 2H), 2.05 (s, 3H). HRMS (TOF MS ES+): m/z calcd for $C_8H_{12}N_3O^+$ $[M+H]^+$ 166.0975, found 166.0971

4-Amino 3-methoxy benzohydrazide (7a) - ^{1}H-NMR (400 MHz, DMSO-d_6): 9.37 (bs, 1H), 7.30 (s, 1H), 7.27 (d, *J* = 8.1 Hz, 1H), 6.60 (d, *J* = 8.1 Hz, 1H), 5.24 (s, 2H), 4.33 (s, 2H), 3.80 (s, 3H). HRMS (TOF MS ES+): m/z calcd for $C_8H_{11}N_3O_2^+$ $[M+H]^+$ 182.0924, found 182.0922.

4-Amino 3-nitro benzohydrazide (8a) - ^{1}H-NMR (400 MHz, DMSO-d_6): 9.68 (bs, 1H), 8.49 (s, 1H), 7.80 (d, *J* = 8.9 Hz, 1H), 7.73 (bs, 2H), 6.99 (d, *J* = 8.9 Hz, 1H), 4.41 (bs, 2H). HRMS (TOF MS ES+): m/z calcd for $C_7H_9N_4O_3^+$ $[M+H]^+$ 197.0669, found 197.0671.

4-Amino 3-chloro benzohydrazide (9a) - ^{1}H-NMR (400 MHz, $DMSO_{d6}$): 9.45 (s, 1H); 7.71 (d, J=2 Hz, 1H); 7.53 (dd, J=8 Hz, J=2 Hz, 1H); 6.75 (d, J=8 Hz, 1H); 5.87 (s, 2H); 4.35 (s, 2H). HRMS (TOF MS ES+): m/z calcd for $C_7H_9ClN_3O^+$ $[M+H]^+$ 186.0429, found 186.0427.

4-Amino 3-bromo benzohydrazide (10a) - ^{1}H-NMR (400 MHz, DMSO-d_6): 9.43 (bs, 1H), 7.85 (d, *J* = 2.0 Hz, 1H), 7.55 (dd, *J* = 8.5, 2.0 Hz, 1H), 6.74 (d, *J* = 8.5 Hz, 1H), 5.80 (bs, 1H), 4.33 (bs, 1H). HRMS (TOF MS ES+): m/z calcd for $C_7H_9BrN_3O^+$ $[M+H]^+$ 229.9924, found 229.9927.

2.3. Peroxidase activity assay

Peroxidase activities was measured by slight modification of following procedure in all purification steps, Increase of absorbance at 470 nm during the oxidation of guaiacol to tetra guaiacol (λ_{max} = 470 nm, ε = 26.6 mM^{-1} cm^{-1}) was used for determination. The reaction mixture contained; a final concentration 15 mM guaiacol, 7.5 mM H_20_2, and 0.03 M phosphate buffer (pH 6.0.). The reaction was initiated by the addition of 2 microliters of enzyme and measurements were recorded at 470 nm for 60 sec-intervals for 3 min. A unit of

peroxidase activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol in 1 min. [22].

2.4. Determination of Inhibition kinetics

The inhibition effects of synthesized molecules (**1a-10a**) on BR-POD were measured to determine the potentials to become a ligand. POD activity was measured in the presence of different concentrations of synthesized molecules (**1a-10a**) for defining the IC_{50} values. Three different inhibitors and five different Guaiacol concentrations were used for determination of K_i values. K_i and the inhibition type were calculated from Lineweaver–Burk graphs (1/V-1/[S]) [23].

2.5. Preparation of affinity gels

Affinity gels were synthesized by according to a published procedure [20] with synthesized molecules (**1a-10a**) that have a reversible inhibition effect. L-Tyrosine was used as a spacer arms for Sepharose 4B, and the molecules (**1a-10a**) were coupled to the Sepharose-4B-L-Tyrosine via the ring-bound amine group on these molecules by diazotization [24]. Steps of affinity gels synthesis were checked with FT-IR.

2.6. Preparation of Enzyme Extracts

Crude extracts of each radish species were prepared to load into affinity columns. For this purpose, 50 g of small cut radish pieces was crushed with liquid nitrogen and homogenized into 100 mL of 300 mM phosphate buffer (pH 7.0.) by using a blender. The homogenate was filtered with Whatman filter paper No. 1 and was centrifuged at 20.000 × g for 15 min. Supernatant was collected and stored -80 °C for using in further purification steps.

2.7. Purification of Peroxidases

The affinity gels were synthesized using 2 g of CNBr activated Sepharose-4B (Sigma C9142) and loaded into 1 * 10 cm columns. Total gel volumes were between 6-7 ml and varied according to the ligand bound. Affinity columns were equilibrated with phosphate buffer (10 mM, pH 6.8). Then, 5-6 ml of homogenates were loaded into the columns and were washed with 25 mM phosphate buffer (pH 6.8). POD enzymes were eluted with 0.5 M $NaClO_4$/0.1 M $NaCH_3COO$ (pH 6.8). Enzymatically active fragments was collected and dialyzed. Protein

concentrations in the homogenates and purification steps were determined by the modified Lowry method [25]. Purification yields were calculated with the following formula:

$Y = EA_1 / EA_2 *100$

*Y: *Yield,* EA_1*: Homogenate Total Activity (EU),* EA_2*: Purified Enzyme Total Activity (EU)*

2.8. SDS-PAGE electrophoresis

Molecular masses of the purified PODs, which isolated from different radish sources, were analyzed by SDS-PAGE (BIO-RAD Mini Protean Tetra System) with polyacrylamide gel (12%) and stacking gel (4%). POD's were detected by the silver staining method and molecular weights were estimated by prestained protein Ladder (Thermo Scientific 26616) [26].

2.9. Determination of Columns Binding Capacity

Columns binding capacities were determined for defining the best purification columns, after purification studies with synthesized affinity columns. The column capacity was measured according to the following procedure: One ml of synthesized gels (**1a, 2a, 3a, 7a, 8a, 9a, 10a** of Sepharose-4B-L-Tyrosine) was poured and transferred to 1x10 cm columns, which equilibrated with equilibration buffer. Then, the columns were saturated with BR-POD enzyme and unbound enzyme was washed with wash buffer. After the washing process, bounded POD enzymes were eluted with the appropriate elution buffer and protein amounts were calculated. The gels were dried, weighed and the POD binding capacity was determined as µg protein / g gel.

3. Results

3.1. Inhibition Studies

BR-POD was purified for determining inhibition effects of the synthesized molecules (**1a-10a**) according to an existing procedure [20]. POD activities were calculated by adding different concentrations of inhibitors to the reaction medium at non-saturated guaiacol concentration. Activity-Concentration (%) graphs were created, and then IC_{50} values were calculated. The results are summarised in Table 1. Inhibition types were determined to set whether or not the synthesized molecules (1a-10a) have potential to be used as ligands. This is

important for purification process of enzymes because only the reversible-inhibitors can be used as ligand. K_i values that indicating interest of the enzyme to the inhibitors were also calculated. K_i values obtained from inhibition studies with BR-POD are presented in Table 1. It was determined that all molecules could be used as a ligand for purification according to their Ki values.

3.2. Synthesis of Affinity Gels and The FT-IR Results

Affinity gel was prepared with the molecules showing reversible inhibition. As shown in Figure 2, 4-amino benzohydrazide derivatives coupled to Sepharose-4B-L-Tyrosine support matrix by diazotization to form the affinity gels. The binding patterns for Sepharose-4B-L-Tyrosine-1a gel were determined from the obtained FT-IR results. As shown in Figure 3, the Sephorase-4B / imidocarbonate structure corresponding to the C-O tension exhibits a weak peak at 858 cm-1 with a broad and intense band at 1380 cm^{-1}, as expected. In addition, the spectrum exhibits a very weak band corresponding to the C = N tension at about 1650 cm^{-1} (a).

Sepharose-4B exhibits a broad band as a result of L-Tyrosine binding symmetrical / asymmetric stretches of phenolic C-OH, seconder amine N-H, aromatic C-H and aliphatic C-H in the range of 3500-2500 cm^{-1} wavelengths respectively. The low frequency region of the spectrum confirms that the phenolic ring and carboxylic acid groups are involved in the structure, 1720, 1640, 1365 and 1050 cm^{-1}, respectively, corresponding to acidic C = O, aromatic C = C and phenolic C-OH stretches (b). Observation of new peaks originating from the N = N and N-NH stretches at 1576, 1429 and 1150 cm^{-1} wave numbers as well as the peaks present in the spectrum confirm that the desired coupling of diazonium salt to sepharose-4B-tyrosine takes place. (c).

3.3. Purification of POD Enzymes by Affinity Chromatography

After the synthesis of affinity gels, homogenates, which amounts of proteins were determined and enzyme activities were measured, were loaded on affinity columns. When 4-amino 2-nitro benzohydrazide (**3a**) were used as ligand, the BR-POD enzyme was purified in 33% yield and 613-fold and The T-POD enzyme was purified in 54.8% yield and 665-fold. While Molecular weight of BR-POD was determined as 47 kDa, Molecular weight of T-POD enzyme was determined as 47 kDa by SDS-PAGE. The SDS-PAGE images are presented in Figures 4a and 4b. 4-Amino 2-nitro benzohydrazide (**3a**) was also found to be the most

effective ligand for purification both of BR-POD and T-POD enzyme. When the 4-amino 3-bromo benzohydrazide (**10a**) was used as ligand, the SR-POD enzyme was purified in 34.4% yield and 781-fold. SDS-PAGE was performed to determine the purity and molecular weights of the SR-POD enzyme Figure 4b. Molecular weight of SR-POD enzyme was determined as 47 kDa. It was also determined that all 4-amino benzohydrazide derivatives could be used as ligand in purification of KR-POD enzyme (Table 2). Especially, 4-amino 3-methyl benzohydrazide (**6a**) molecule has achieved significant success in purification of the enzyme and this molecule enabled to purification of the enzyme in 41% yield and 282-fold. Molecular weight of the enzyme was found to be 47 kDa (Figure 4c). 4-amino 2-methyl benzohydrazide (**1a**) and 4-Amino 2-methoxy benzohydrazide (**2a**) were determined to be most effective ligands in purification of D-POD enzyme. Purification factors of D-POD enzyme were recorded as 22.7% yield 595-fold and 17.8% 175-fold, respectively. Molecular weight of the enzyme was found to be 48.5 kDa (Figure 4d). All purification results are presented in Table 2 and the detailed purification results of 4-amino 3-bromo benzohydrazide (**10a**) are presented in Table 3.

3.4. Column binding capacity studies

Effects of the temperature, pH and ionic strength on column binding capacity were investigated for BR-POD enzyme. Binding capacity was defined as protein/gram gel. The column binding capacity of 4-Amino 3-bromo benzohydrazide (10a) was determined as 1353.3 µg/g gel at the conditions 15 °C, pH 7.5 and 0.3 M ionic strength. The optimal conditions of the ligands for purification of the BR-POD enzyme are presented in Table 4.

4. Discussion

Peroxidases are enzyme families that are used in industry and they have commercial importance. Peroxidase enzymes are used in many bioprocesses such as cleaning of phenolic compounds from wastewaters, removing of synthetic dyes, clinical diagnosis and organic synthesis reactions [1, 27]. Because of the common utilizations, studies on purification of the peroxidases are increasingly important. Many researchers have been working on purification of peroxidases by different chromatographic methods. Among these methods, affinity technique provides superiority to other techniques because it is short-term renewable, one-step and cheap. In previous studies, POD was purified from papaya, spinach leaves, black radish, turnip, daikon, orange peels, red cabbage and orange in 30.22 fold with a yield of

44.37% in three chromatographic steps [28], 22.6-fold with a yield of 22.6 % in two chromatographic steps [29], 40.3-fold with a yield of 10.6% in single chromatographic step [20], 269-fold with a yield of 9% in single chromatographic step [20], 60-fold in two chromatographic steps [30], 18-fold with a yield of 94% in two chromatographic steps [31], 120-fold with a yield of 120% in single chromatographic step [32] and 2.5-fold with a yield of 82.5% in two chromatographic steps [3], respectively.

According to our knowledge, many chromatographic methods and their combinations have been used in the purification of the peroxidase enzymes. While yields vary ranged from 94% to 9%, purification folds vary ranged from 2.5 to 269. In the studies that obtained high yield, the purification fold generally decreased and high purification folds also caused low yields. It is desired to be high both of purification folds and yields for an effective purification method. In this study, the peroxidase enzymes from different radish species were purified in yields of more than 30% and purification folds of more than 500. Single-band was observed in SDS-PAGE from purified POD enzymes. It was shown that 4-amino benzohydrazide derivative molecules were only specific to peroxidase enzyme and the molecules did not interact with other proteins in homogenates.

Structural differences in synthesized derivatives are an important factor effecting enzyme interaction. Both of the substituent (-CH_3, -Cl, -Br, -OCH_3, -NO_2) and the position to which it was binding (2 or 3) significantly changed the purification fold and yield. -NO_2 and -Br functional groups bound to the ring increased the efficiency of the purification. (See Table 2, 3a, 10a). -Cl bonded to position 3 decreased the affinity of the ligand for D-POD and reduced the yield by up to 3%. OCH_3 was more effective in positions 2 than position 3. While CH_3 on the position 3 was very effective in the purification of KR-POD, it did not show the same effect in the purification of other PODs. As shown in Table 2, the effectiveness of purification is related to the type of functional group, the binding position and the type of peroxidase.

Detailed kinetic studies were conducted for the synthesized molecules (**1a-10a**) and these molecules were defined as new inhibitors of the peroxidase enzymes. Additionally, SR-POD and KR-POD enzymes were purified with this study first time, successfully.

5. Conclusions

In order to purify quickly, cheaply and in high yield the peroxidase enzymes from radish species, an affinity chromatography technique was developed on the Sepharose-4B-L-tyrosine carrier matrix by using 4-amino benzohydrazide derivatives as ligands. In particular, it was

achieved that high yields and purity in purification by using 4-amino 3-bromo and 4-amino 2-nitro benzohydrazide molecules. This method is superior to existing methods in terms of yield and purification factor. In addition, it significantly shortens the time spent in obtaining POD enzymes.

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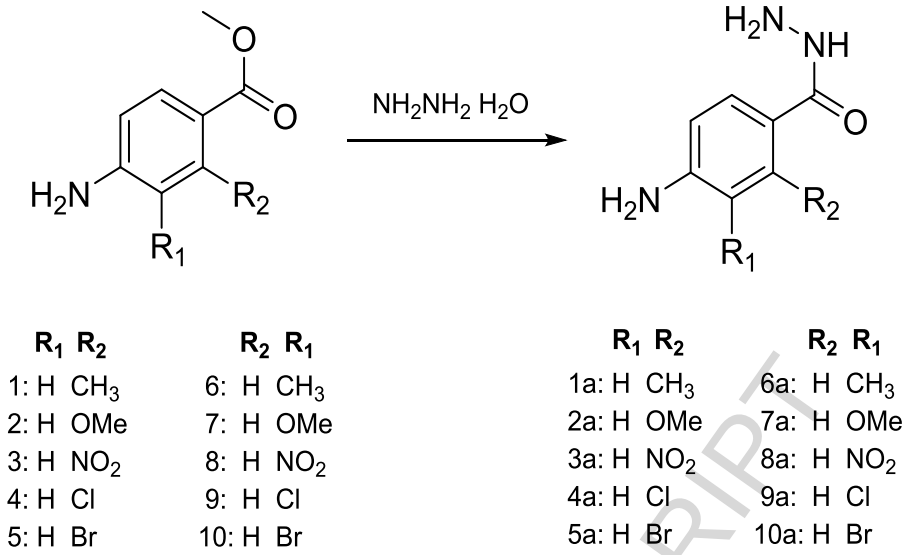


R_1	R_2
1: H	CH_3
2: H	OMe
3: H	NO_2
4: H	Cl
5: H	Br

R_2	R_1
6: H	CH_3
7: H	OMe
8: H	NO_2
9: H	Cl
10: H	Br

R_1	R_2
1a: H	CH_3
2a: H	OMe
3a: H	NO_2
4a: H	Cl
5a: H	Br

R_2	R_1
6a: H	CH_3
7a: H	OMe
8a: H	NO_2
9a: H	Cl
10a: H	Br

Figure 1. Synthesis route of 4-amino benzohydrazide derivatives (**1a**-**10a**)

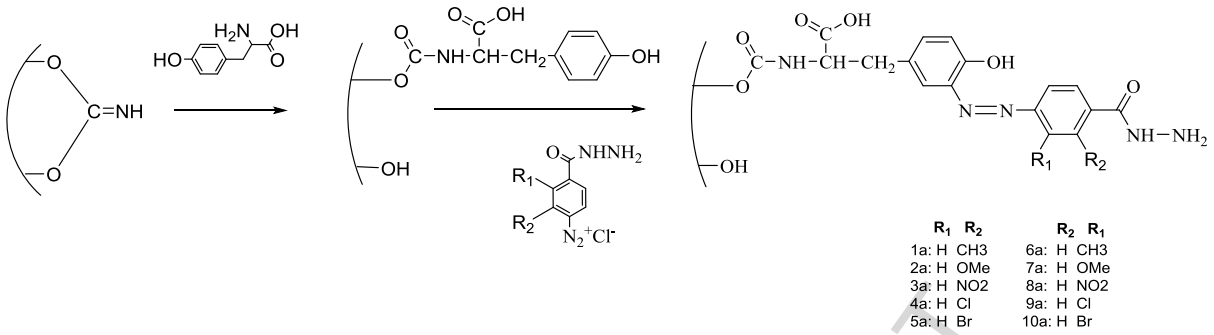


Fig 2. Synthesis of the Sepharose-4B-L-Tyrosine-(**1a-10a**) affinity gels.

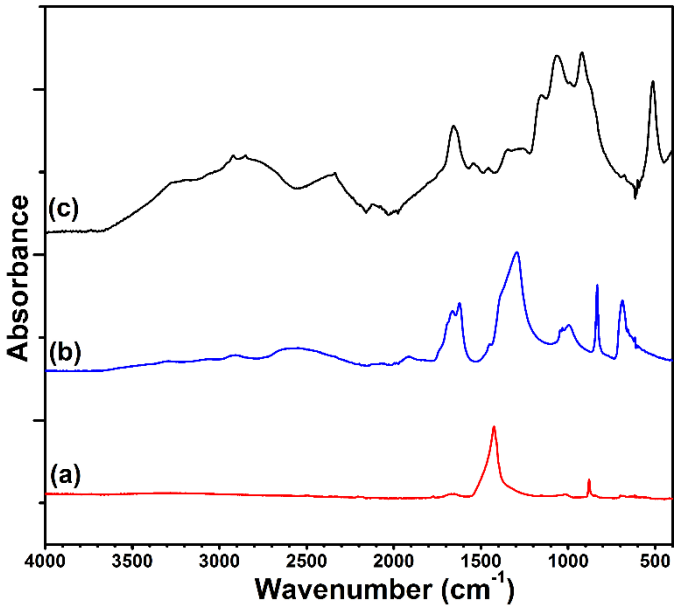


Fig 3. FTIR (Burker VERTEX 70V) results of **a**: Sepharose-4B imidocarbonate, **b**: Sepharose 4B-L-Tyrosine, **c**: Sepharose 4B-L-Tyrosine-4-Amino 2-methyl benzohydrazide (**1a**) affinity gel.

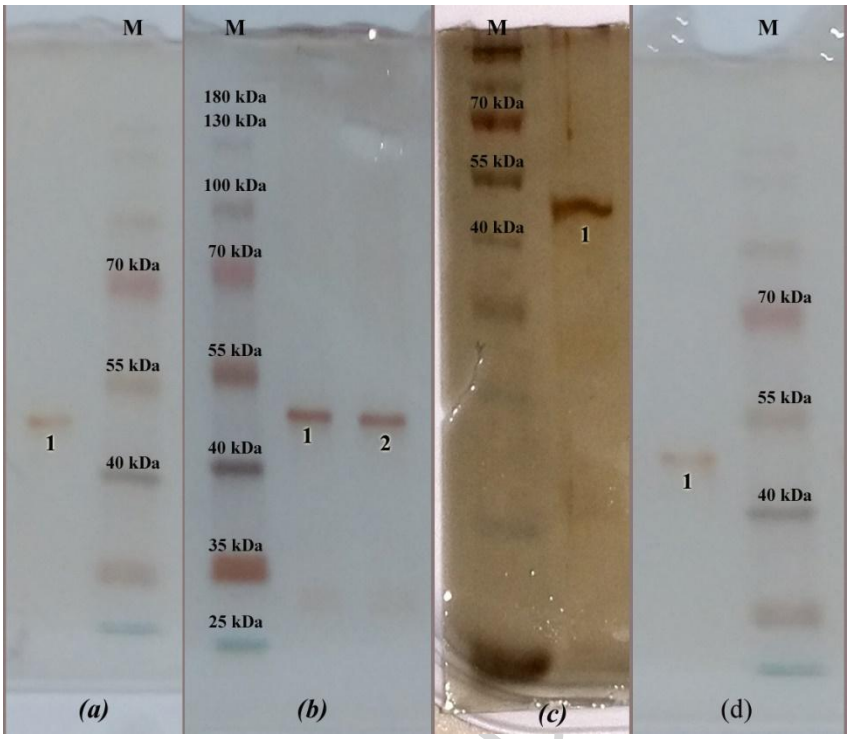


Fig 4. SDS-PAGE bands of T-POD, BR-POD, D-POD, SR-POD and KR-POD Enzymes purified from Sepharose 4B-L-Tyrosine-4-amino 3-bromo benzohydrazide affinity jel. M: Markers (Thermo Scientific 26616). **(a)**; 1: BR-POD, **(b)**; 1: T-POD, 2: SR-POD, **(c)**; 1: KR-POD, **(d)**; 1: D-POD.

Table 1. IC_{50} value, K_i constant and inhibition type of 4-amino benzohydrazide derivatives for BR- POD.

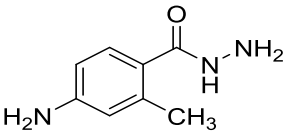
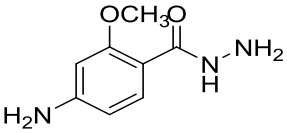
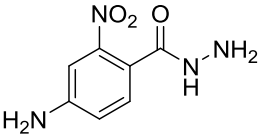
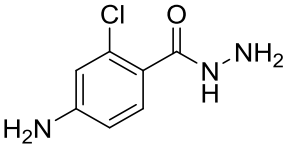
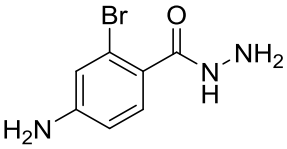
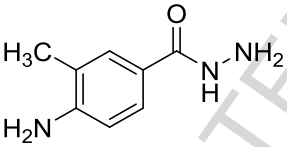
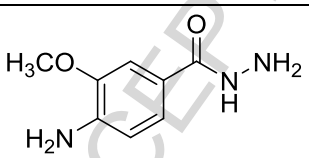
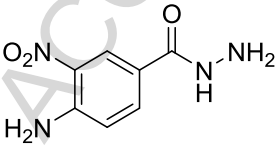
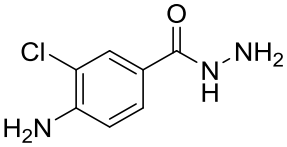
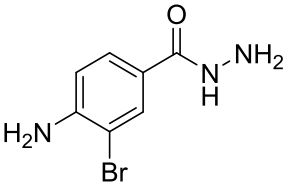
Inhibitors		IC_{50} (mM)	K_i (mM)	Inhibition type
1a		0,371	0,415 ± 0,0862	Non-competitive
2a		0,231	0,271 ± 0,019	Non-competitive
3a		0,961	1,04 ± 0,0649	Non-competitive
4a		0,346	0,356 ± 0,055	Non-competitive
5a		0,373	0,547 ± 0,081	Competitive
6a		0,000124	0,00015 ± 0,000032	Non-competitive
7a		0,086	0,103 ± 0,039	Non-competitive
8a		0,440	0,356 ± 0,116	Non-competitive
9a		0,173	0,047 ± 0,017	Competitive
10a		0,115	0,127 ± 0,006	Non-competitive

Table 2. Purification results of POD enzymes from radish species with Sepharose-4B-L-tyrosine-(1a-10a) affinity gels.

Ligand	BR-POD		T-POD		SR-POD		KR-POD		D-POD	
	% Yield	Fold	% Yield	Fold	% Yield	Fold	% Yield	Fold	% Yield	Fold
1a	14,1	211	11,7	444	20,2	591	13	581	**22,7**	**595**
2a	33,6	676	20,1	806	30,2	322	6,8	262	17,8	175
3a	**33,8**	613	**54,8**	665	14,9	678	21,3	705	10	363
4a	14,5	263	8,1	292	6	273	15,3	582	4,3	342
5a	28,5	258	18,2	330	26,5	601	24	424	16,5	130
6a	14,2	191	10,2	234	15,8	103	**40,9**	282	5,91	291
7a	10,1	244	12,2	653	9,1	412	16,3	337	13	557
8a	7,5	365	10,1	403	6,1	502	21	**745**	4,7	371
9a	19,5	248	13,6	676	4,6	236	9,9	378	3	156
10a	31,4	**677**	22	**1348**	**34,4**	**781**	13,8	245	9,8	388

Table 3. The detailed purification results of Sepharose 4B-L-Tyrosine-4-amino 3-bromo benzohydrazide affinity gel

SOURCE	STEP	Total Volume (mL)	Activity (EU/mL)	Protein (mg/mL)	Total Activity (EU)	Total Protein (mg)	Specific activity (EU/mg)	Purification Yield %	Purification Fold
Black radish	[a]**Step1**	5	108	1,45	540	7,25	74,5	100	1
	[b]**Step2**	3	56,5	0,0011	169	0,003	50454	31,4	677
Daikon	[a]**Step1**	6	39,2	1,18	235,2	7,08	33,2	100	1
	[b]**Step2**	3	7,74	0,0006	23,2	0,0018	12900	9,86	388,5
Kohlrabi radish	[a]**Step1**	6	140,4	1,24	842	7,4	113,2	100	1
	[b]**Step2**	3	38,9	0,0014	116	0,004	27785	13,8	245,4
Sweet radish	[a]**Step1**	6	51,7	1,02	310,2	6,12	50,6	100	1
	[b]**Step2**	3	35,6	0,0009	106,8	0,0027	39555,5	34,4	781,7
Turnip	[a]**Step1**	5	148	1,29	740	6,45	114,7	100	1
	[b]**Step2**	1,5	108,2	0,0007	162,3	0,001	154571	22	1348

[a]**Step1**: Crude Homogenate

[b]**Step2**: Sepharose 4B-L-tyrosine-4-amino benzohydrazide affinity column chromatography

Table 4. Optimal parameters of affinity gels for the purification of BR-POD enzyme

Ligand	Optimum pH	Optimum Temperature	Optimum Ionic strength	Binding capacity (µg/g dry gel)	Loaded BR-POD Samples (mg/g dry gel)	Recovery (%)
1a	8	15	0,3	223,2	4	5,5
2a	8	15	0,35	1400	4	35
3a	7	20	0,3	2660	4	56,5
7a	7	25	0,4	1916,6	4	50
8a	7,5	15	0,4	751,06	4	18,8
9a	7,5	10	0,4	573,3	4	14,3
10a	7,5	15	0,3	1353,3	4	33,8

Highlights

- 10 different 4-amino benzohydrazide derivatives were synthesized and identified as new POD inhibitors.
- These derivatives were coupled to Sepharose 4B-L-Tyrosine support matrix to form affinity gels.
- The binding patterns for Sepharose-4B-L-Tyrosine-(1a-10a) affinity gels were controlled by FT-IR.
- The radish peroxidases were purified in a single step with the prepared affinity gels.