Physicochemical properties of free and immobilized tyrosinase from different species of yam (*Dioscorea spp*)

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A R T I C L E   I N F O

A B S T R A C T

A shortened method of purification and immobilization of tyrosinase from different species of yam (*Dioscorea spp*) on insoluble supports is described. The enzyme was purified by aqueous two-phase partitioning (ATPS) followed by gel filtration chromatography. The purified enzyme was immobilized on Ca-alginate, polyacrylamide gel or as cross-linked enzyme aggregate (CLEA) to obtain a yield of between 51–64%, 33–46% and 52–65% respectively for all the yam species. The optimum pH obtained for tyrosinase immobilized on polyacrylamide gel and CLEA was equivalent to that of free enzyme (pH 6.5). In contrast, Ca-alginate entrapped tyrosinase exhibited a shift of optimum pH to 7.0. Entrapped Tyrosinase in polyacrylamide gel and Ca-alginate also retained the same optimum temperature as the free enzyme (50 °C). While the optimum temperature of CLEA shifted to 60 °C. When subjected to four repeated use cycles, tyrosinase entrapped in polyacrylamide gel, Ca-alginate and CLEA still retained close to 40, 35 and 45 % of their initial activities respectively after the fourth cycle. The overall result further suggests yam tyrosinase as a promising enzyme for biocatalysis and biotechnological applications.

1. Introduction

Tyrosinases are metallo-enzymes containing a type-3 copper-center. They are found in diverse organisms such as fungi, plants, bacteria and mammals [1]. They are bifunctional polyphenol oxidases (PPOs) which employ molecular oxygen for the oxidation of various monophenols to o-diphenols (cresolase activity), and further oxidative conversion of o-diphenols to the corresponding *o*-quinones (catecholase activity) [2,3]. Tyrosinase has been isolated from many fungal and bacterial species including *Agaricus bisporus* [4,5], *Streptomyces castaneoglobisporus* [6] and *Pseudomonas putida* [7].

The enzyme has also been studied in fruits and vegetables ranging from apple [8], Blanquilla pear [9], cabbage [10], lettuce [11] and Loquat fruit [12]. Tyrosinases perform several functions in these organisms which include pigmentation, sclerotization, and defense. Tyrosinase activities (monophenolase and diphenolase) have been exploited in many applications such as production of L-DOPA in pharmaceuticals, waste-water detoxification in environmental technology, building of biosensors for detection of compounds-containing phenols and modification of food proteins

via cross-linking effects [13]. Tyrosinases play an important role in the regulation of the oxidation-reduction potential of cell respiration and in wound healing in plants [14].

Tyrosinase is the initiating and rate-limiting enzyme in the melanin biosynthetic pathway, and therefore the prime target for the design of antimelanogenic compounds [15].

Isolation and purification of tyrosinase is a process known over a long time and well developed. Some of these purification processes are associated with loss of enzyme activity and low level of purity [16]. This constrains their utilization as biocatalysts, usually when exposed to chemical or physical processes [17]. As a means of improving this circumstance, purification strategies that improve the activity and stability of the resultant enzyme preparations have been developed. These include use of stabilizing agents like alcohols and carbohydrates, chemical modification, or use of mutant proteins [18]. Some of these processes are laborious and time-consuming, yet associated with loss of enzyme activity. Using aqueous two-phase partitioning (ATPs) during purification gives several benefits such as environmental biocompatibility, reduced process time and improved properties of the resulting enzyme molecules. For these reasons, it has been deployed in this investigation.

Immobilization is one of the most important and widely used techniques in biotechnology, in which catalysts are attached to a solid support that is insoluble in the reaction mixture [19]. It offers distinct advantages such as operational stability, ease of product

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recovery and low enzyme production cost as the immobilized enzymes can easily be separated from reaction medium and hence used continuously [20]. Several workers have immobilized tyrosinase on different supports including covalent attachment to cellulose [21], entrapment in polyacrylamide gel [22], separation by a nylon membrane [23], entrapment in zeolite [24], in chitosan [25], in alginate gel [26], by binding the enzymes to magnetic beads [27] or by formation of cross-linked tyrosinase aggregate [28]. However, with tyrosinase, few of these immobilization approaches have been successful. In addition, most of the previous workers have not been able to compare the relative advantage(s) of immobilization on different supports in order to provide information on the immobilization efficiency for suitability in several biotechnology and biocatalysis applications.

In the present work, we focused on shortening the purification scheme to achieve an efficient separation with concomitant improvement in stability and catalytic activity together with comparative efficiency of immobilization of the enzyme on different insoluble media.

2. Materials and methods

2.1. Materials

Four (4) yam tubers, from *Dioscorea alata*, *Dioscorea praehensilis*, *Dioscorea rotundata* and *Colocasia esculenta* were obtained from farms in Ile-Ife environs, Southwestern Nigeria. Authentication of the yam cultivars was carried out at IFE Herbarium, Botany Department of Obafemi Awolowo University, Ile-Ife, Nigeria.

2.1.1. Chemicals

Polyethylene glycol (PEG 6000), ammonium sulphate, sodium chloride, lysozyme, horse radish peroxidase, α-chymotrypsinogen A, acrylamide, ovalbumin, bovine serum albumin, N,N,N^1,N^1-tetramethylethylenediamine (TEMED), ammonium persulphate, sodium dodecyl sulphate (SDS), Coomassie brilliant blue R-250, sodium alginate, glutaraldehyde and 3,4-dihydroxyphenyl-L-alanine (L-DOPA), were obtained from Sigma Chemical Company, St Louis, USA. Protein markers for SDS-PAGE were obtained from Carl Roth, Karlsruhe, Germany. Sephadex G-100 was obtained from GE Healthcare Bio-sciences (GHB), Uppsala, Sweden. Other reagents used were of analytical grade.

2.2. Methods

2.2.1. Preparation of yam homogenates

Homogenates (30 %) of yam tubers were prepared in 0.05 M Na-phosphate buffer, pH 6.5 on ice (4 °C) and then centrifuged using a cold centrifuge at 10,000×g for 30 min. The clear supernatants obtained were thereafter assayed for tyrosinase activity using 3,4-dihydroxy-L-phenyalanine as substrate.

2.2.2. Assay for tyrosinase activity

The assay was carried out according to the method of Lerch and Etlinger [29] as modified by Ilesanmi et al. [30]. Briefly, 1 mL assay mixture in a cuvette contained assay buffer (0.05 M phosphate buffer, pH 6.5), 1 mM L-DOPA and appropriate enzyme volume. A change in absorbance of the assay mixture was then monitored in a spectrophotometer. The assay mixture in which distilled water was used to replace enzyme served as blank. Absorbance was read at intervals of 15 s at 475 nm and the initial rate of the reaction was calculated as the change in absorbance per minute.

A unit of tyrosinase activity is the amount of enzyme that catalysed the formation of 1 μmole of o-dopaquinone per minute under the standard assay condition ($\varepsilon_{475} = 3600$ M^{-1} cm^{-1}).

2.2.3. Protein concentration determination

The determination of protein concentration of tyrosinase at each purification was carried out as described by Bradford [31] using BSA as standard.

2.2.4. Purification of yam tyrosinase

The enzymes were purified using aqueous two phase separation (ATPS) with gel filtration chromatography (Sephadex G-100) as stated below.

2.2.4.1. Purification by aqueous two-phase partitioning.
The ATPS system contained 2% w/v NaCl, 7.5 % w/v (NH$_4$)$_2$SO$_4$ and 24 % w/v PEG 6000 along with the crude enzyme. The mixture was stirred continuously for 2 h at 4 °C until the salts were completely dissolved in the crude enzyme preparation. The system was left for 6 h at 4 °C to achieve phase separation. (NH$_4$)$_2$SO$_4$ caused the phase separation by flotation. The specific activities of the enzyme in both phases were calculated. After separation, the enzyme-rich phase was subjected to dialysis against Na-phosphate buffer (0.05 M, pH 6.5) for 6 h at 4 °C to remove other salts. All partition experiments were performed in triplicate.

2.2.4.2. Purification using size-exclusion chromatography (Sephadex G-100).
The enzyme solution from ATPS for each of the cultivars was further purified separately on column (1.0 × 40 cm) packed with Sephadex G-100. The column was washed with distilled water and equilibrated with homogenization buffer before layering of enzyme. Forty (40) fractions were collected, 1 mL each at a rate of 12 mL/h. The fractions were analysed and active regions were pooled and freeze-dried. The lyophilized samples were re-dissolved in small volume of buffer and stored for subsequent use. The native and subunit molecular weights of the purified enzyme were estimated by interpolation of partition coefficient (K_{av}) values from standard curve on calibrated Sephadex G-100 and relative mobilities (R_m) values from curve on 12 % SDS-PAGE respectively. The SDS-PAGE was carried out according to the method of Laemmli [32].

2.2.5. Stability of free tyrosinase in organic media

The enzyme solution from the yam species was incubated in different concentration (0–70 %) of DMSO, ethanol, methanol, acetone, chloroform and ether at 30 °C for 24 h. The residual activities were thereafter measured. The activities on L-DOPA without solvent were taken as 100 % activity.

2.2.6. Kinetic constants determination for the free enzymes

The effect of varied concentration of L-DOPA between 1 and 30 mM in 0.05 M Na-phosphate buffer (pH 6.5) on activities of tyrosinase was determined. Kinetic constants were estimated from the data obtained using non-linear regression on Graph Pad Prism 5.

2.2.7. Immobilization of free tyrosinase in alginate gel

The method of Palmieri et al. [33] was employed. 0.4 g of Na-alginate was dissolved in 20 mL of the enzyme preparation, to obtain a 2% enzyme-sodium alginate solution. It was then extruded drop wise into a beaker containing 100 mL of 200 mM calcium chloride at 4 °C. After 2 h, the beads formed were rinsed with distilled water and stored in 100 mM sodium-acetate buffer, pH 6.0 for subsequent use.

2.2.8. Immobilization of free tyrosinase by entrapment in polyacrylamide gel

Entrapment of tyrosinase in polyacrylamide gel was carried out according to the method described by Skrylabin and Koshcheenko [34]. Acrylamide gel solution prepared from 9% w/v acrylamide and

1% w/v bisacrylamide in 50 mM Na-phosphate buffer, pH 6.5 was mixed with the enzyme preparation from the yam species in the ratio of 1:2. 10 mL of 0.5 % ammonium persulfate and 0.6 mL of 50 % TEMED were then added to the mixture, with gentle stirring. It was then poured into different petri dishes for setting of the gel.

2.2.9. Cross-linked enzyme aggregate (CLEA) preparation

Cross-linked tyrosinase aggregate (CLEA) was prepared as described by Xu et al. [28]. It involved progressive addition of ammonium sulfate to the tyrosinase preparation to reach 90 % saturation. Upon stirring of the mixture for 10 min at 4 °C, 0.5 % glutaraldehyde (25 % wt.) was added dropwisely and subjected to further stirring at 4 °C for 16 h. The mixture was then centrifuged to recover pellets. The pellets were thereafter washed with 50 mM Na-phosphate buffer, pH 6.0 and allowed to dry before use.

2.2.10. Properties of the immobilized enzymes

The properties investigated were optimum pH, optimum temperature, operational stability, and reaction time course. Tyrosinase activities of the immobilized enzyme preparations were measured at varying pH values ranging from 3−10. The effect of temperature on tyrosinase activities were measured at temperatures variations from 10 to 80 °C. For the reaction time course measurements, 0.1 g of Ca-alginate beads, 0.1 g of polyacrylamide gel and 50 mg of CLEAs were incubated in substrate solution containing 10 mM L-DOPA dissolved in 0.0 5 M phosphate buffer,

pH 6.5. Aliqouts were taken at 1 h interval for 7 h to monitor the formation of dopachrome.

Operational stability of the immobilized enzymes was ascertained by repeated use for a total of four (4) cycles. For every cycle, change in absorbance was monitored for 15 min under standard assay conditions and dopachrome formed per minute was calculated. After each cycle, the enzymes were washed severally with 50 mM phosphate buffer, pH 6.5, dried and kept on ice for the next reaction cycle at 2 h intervals.

2.2.11. Statistical analyses

All experiments were done in triplicate and the data are reported as mean ± standard deviation. Other statistical analysis was performed with Graph Pad Prism 5, version 5.01.

3. Results

3.1. Enzyme purification

Rapid purification of the tyrosinase from each species of yam was achieved through aqueous two-phase partitioning by removal of contaminants, non-target proteins and other hydrophobic compounds. Hence, the resulting polyphenol free and concentrated protein solution was found suitable for the subsequent chromatographic purification step. A last purification on gel filtration gave a single activity peak for tyrosinase from each yam species (Fig. 1) resulting to recovery of 59, 54, 55 and 59 % and
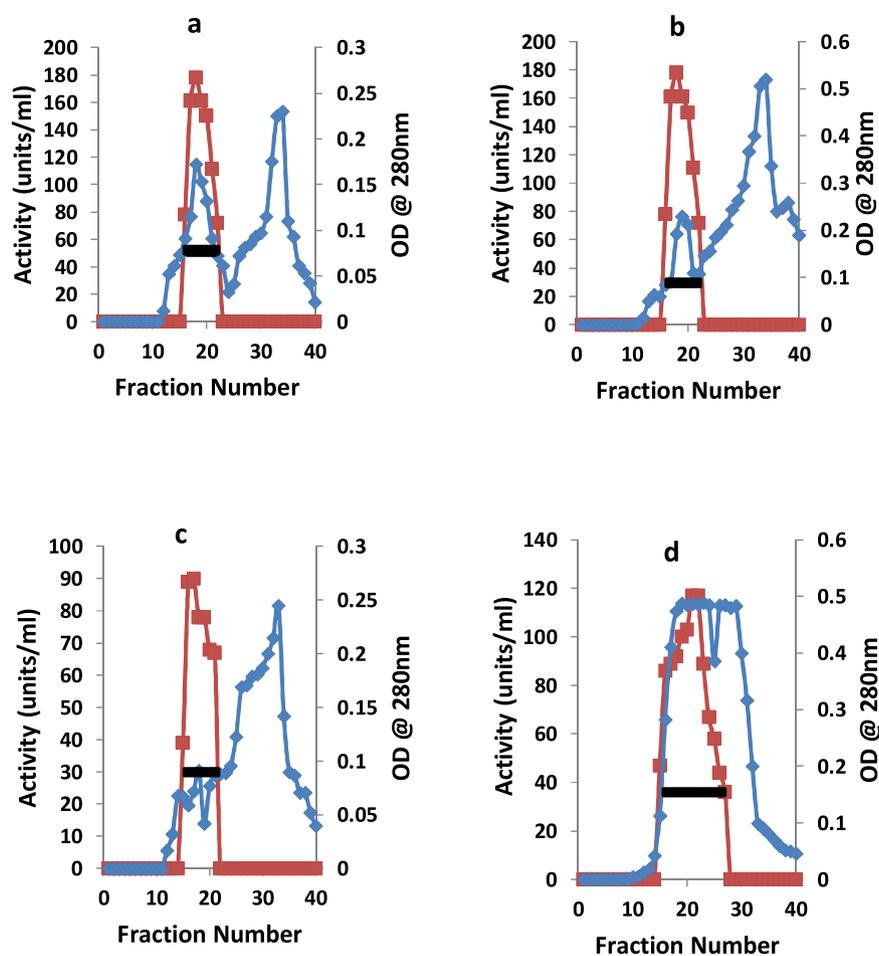

Fig. 1. Elution profile of ATPS-purified tyrosinase on Sephadex G-100. The ATPS pool obtained from (A) *D. praehensilis* (B) *D. alata* (C) *D. rotundata* (D) *C. esculenta* were layered on Sephadex G-100 column (1.0 cm × 50.0 cm). The proteins were eluted with the equilibration buffer (50 mM phosphate buffer, pH 6.5) at a flow rate of 12 mL/h. Fractions of 1 mL each were collected and active fractions were pooled and concentrated. Fractions pooled (▬▬), Absorbance @ 280 nm (◆—◆—◆), Tyrosinase activity (units/mL) (▬■▬).

Table 1
Purification summary of tyrosinase from different yam species.

| Sample	Steps	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)	% Yield	Purification fold
D. praehensilis	Crude	30	166,680	92	1812	100	1
	ATPS	21	180,550	20	9028	108	5
	SEC	10	91,650	6	15,275	55	9
D. alata	Crude	30	156,960	12	13,080	100	1
	ATPS	22	134,176	2.6	52,320	86	4
	SEC	10	93,296	0.8	112,488	59	8.6
D. rotundata	Crude	30	161,120	92	1751	100	1
	ATPS	20	200,010	25	8000	124	4.5
	SEC	10	94,815	6	15,759	59	9
C. esculenta	Crude	30	48,080	164	293	100	1
	ATPS	19	52,728	30	1758	110	6
	SEC	10	28,197	9.4	3000	54	10

*SEC – Size-exclusion Chromatography.
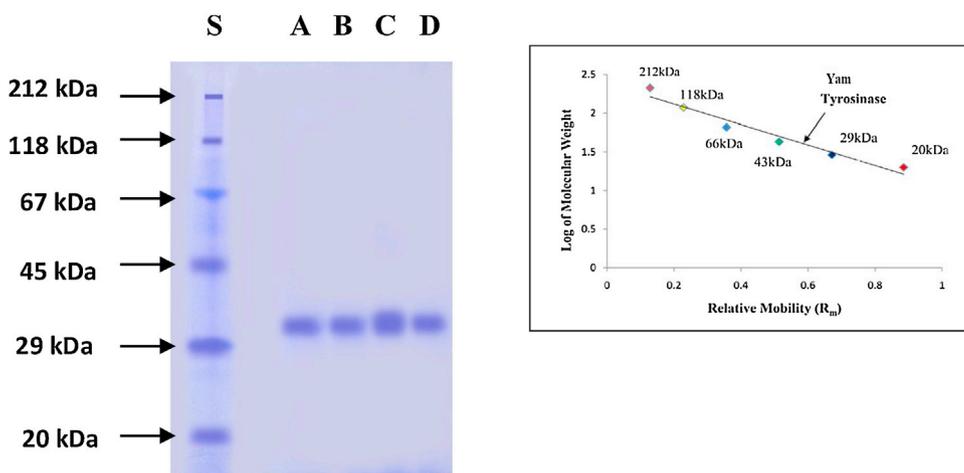
Fig. 2. SDS-PAGE of purified tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta*. Standard proteins: Myosin (212 kDa), β-Galactosidase (118 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20 kDa) are shown on lane S. Purified tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* are shown on lanes A, B, C and D respectively. Inset: Standard plot for the determination of molecular weights of purified tyrosinase from yam species.

Table 2
Kinetic parameters of tyrosinase purified through phase partitioning.

Species	K_m (mM)	V_{max} (units/mg protein)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
D. praehensilis	9.6 ± 3.7	$45,050 \pm 7738$	4.5×10^4	4.8×10^6
D. alata	20.0 ± 11	$215,319 \pm 5625$	21.9×10^4	7.3×10^6
D. rotundata	6.8 ± 2.6	$17,208 \pm 2508$	1.7×10^4	2.6×10^6
C. esculenta	23.7 ± 14	7777 ± 3003	7.1×10^3	3.0×10^5

purification fold of 8.6, 10, 9.1 and 9.3 for *D. alata*, *C. esculenta*, *D. praehensilis* and *D. rotundata* enzymes respectively (Table 1). Single band on SDS-PAGE (Fig. 2) showed that the enzyme preparations were apparently pure, indicating the adequacy of the purification scheme.

3.2. Kinetic and physicochemical parameters of free tyrosinase preparations

The first order rate constants, k_{cat}/K_m of purified tyrosinase were 7.3×10^6 $s^{-1}M^{-1}$, 3.0×10^5 $s^{-1}M^{-1}$, 4.8×10^6 $s^{-1}M^{-1}$, and 2.6×10^6 $s^{-1}M^{-1}$ for *D. alata*, *C. esculenta*, *D. praehensilis* and *D. rotundata* enzymes respectively (Table 2). The native molecular weights of 61.0 ± 2.0, 55.0 ± 2.0 kDa, 61.0 ± 1.0 and 61.0 ± 2.0 were obtained for free tyrosinase from *D. alata*, *C. esculenta*, *D. praehensilis* and *D. rotundata* respectively. Subunit molecular weight of 41.4 ± 0.2, 41.4 ± 0.1 kDa, 41.4 ± 0.1 and 41.4 ± 0.2 were obtained for *D. alata*, *C. esculenta*, *D. praehensilis* and *D. rotundata* respectively on SDS-PAGE.

3.3. Stability of free tyrosinase in organic solvents

Up to 45 % activity was retained by tyrosinase from *D. alata* in 50 % ethanol. Tyrosinase from *D. Praehensilis* retained up to 33 % activity in 50 % methanol. In summary, all the ATPS-purified tyrosinase were stable up to 40, 60, 40 and 50 % in DMSO, ethanol, acetone and methanol respectively. The activity of the enzymes increased in ether between 0–40% (Fig. 3). The activation and stability were retained when incubated 24 h in ≤40 % ether and retained close to 60 % residual activity in 70 % ether.

3.4. Enzyme entrapment in gels and CLEA formation

In polyacrylamide gel, the activity yield, was influenced by acrylamide concentration. Maximum activity was obtained at 10 % acrylamide concentration. In CLEA preparation, higher concentration of glutaraldehyde, led to excessive crosslinking and loss of enzyme activity. 0.5 % final concentration of glutaraldehyde gave
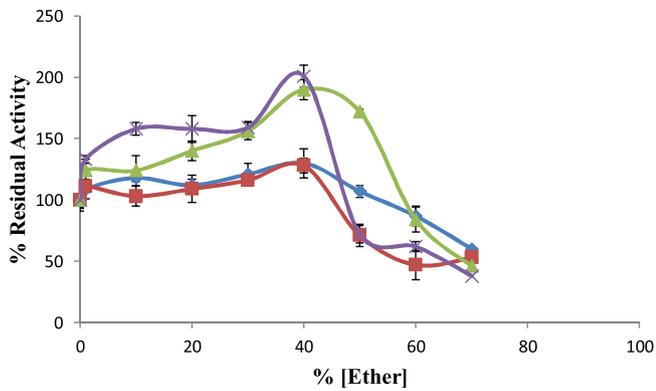
Fig. 3. Stability of purified tyrosinase from *D. praehensilis* (━◆━), *D. alata* (━■━), *D. rotundata* (━▲━) and *C. esculenta* (━✕━) in the presence of ether. The effect of ether on the stability of tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* were investigated by incubating the enzyme solution at 30 °C for 24 h in various concentrations of ether ranging from 0 to 70 % and followed by the measurement of the residual activity. The activity of the enzyme in buffer or organic solvents before incubation was taken to be 100 %.

the highest activity yield. The activity yield obtained for each of the purified enzyme immobilized on Ca-alginate, polyacrylamide gel and CLEA were between 51–64 %, 33–46 % and 52–64 % respectively.
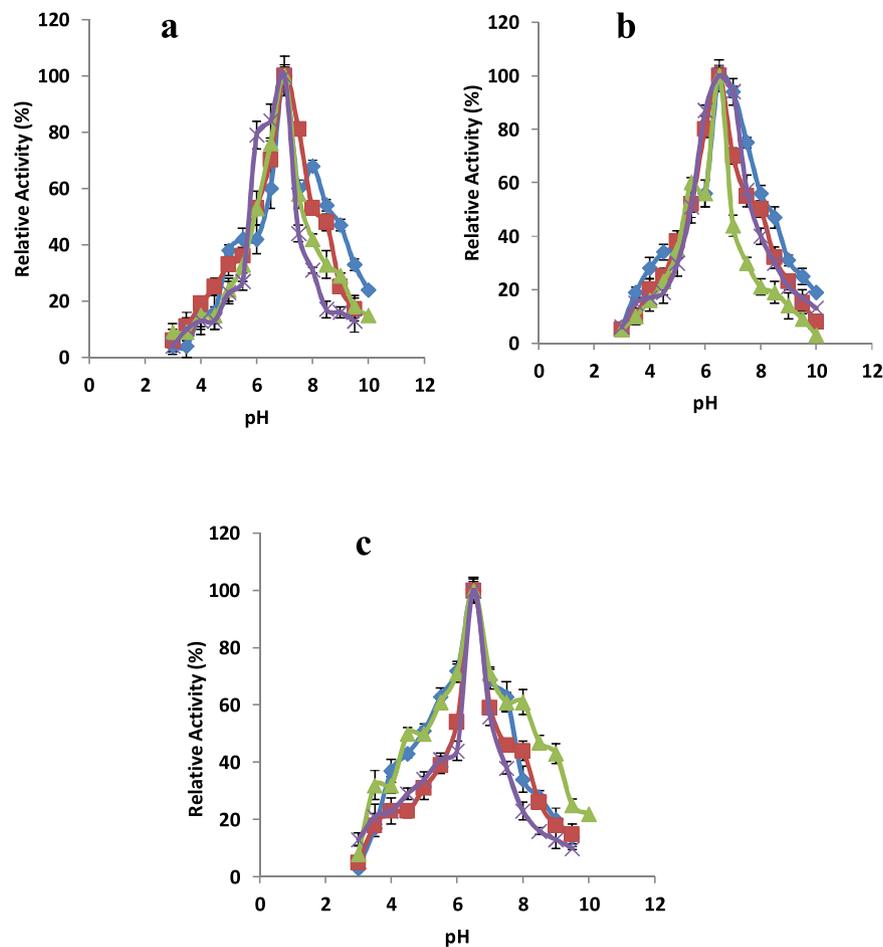
3.5. Properties of immobilized Dioscorea tyrosinase

3.5.1. Optimum pH and temperature

The pH-activity profiles of immobilized tyrosinase in Ca-alginate, polyacrylamide gel and CLEA from all the yam species are shown in Fig. 4. The optimum pH obtained for tyrosinase in polyacrylamide gel and CLEA was respectively 6.5 which were the same as what was obtained for free enzyme preparations. The optimum pH obtained for the enzyme entrapped in Ca-alginate was 7.0, showing a shift from that of free enzyme by 0.5 unit. Loss of activity was observed for the immobilized yam tyrosinase at pH of 5 or below and pH of 8 or above was observed for the immobilized enzymes from all the yam species studied. Tyrosinase entrapped in Ca-alginate and polyacrylamide gel maintained optimum temperature of 50 °C which was the same as that of the soluble enzyme, while there was a shift to 60 °C for the CLEA preparations (Fig. 5). At 70 °C, more than 60 % activity was retained for the tyrosinase entrapped in polyacrylamide gel and CLEA, whereas a progressive decline in activity was noticeable for Ca-alginate entrapped enzymes at temperatures above 50 °C.

3.6. Operational stability (reuseability) of immobilized yam tyrosinase

Table 3 is a summary of reuseability of the immobilized tyrosinase preparations from all the yam species. The activity diminished in progression with number of repeated uses. However, Ca-alginate entrapped tyrosinase from *D. praehensilis* retained 113

Fig. 4. Influence of pH on activity of immobilized *Dioscorea* tyrosinase.
(a) Ca-alginate (b) polyacrylamide gel (c) CLEAs. Activity of the immobilized tyrosinase from *D. praehensilis* (━◆━), *D. rotundata* (━■━), *D. alata* (━▲━) and *C. esculenta* (━✕━) was measured at different pH values ranging from 3 to 10 at room temperature. The following buffer systems at the indicated pH ranges were used: 50 mM citrate buffer, pH 3.0–5.0; 50 mM MES buffer, pH 5.5–6.5; 50 mM HEPES buffer, pH 7.0–8.5; and 50 mM glycine–NaOH buffer, pH 9.0–10.0.
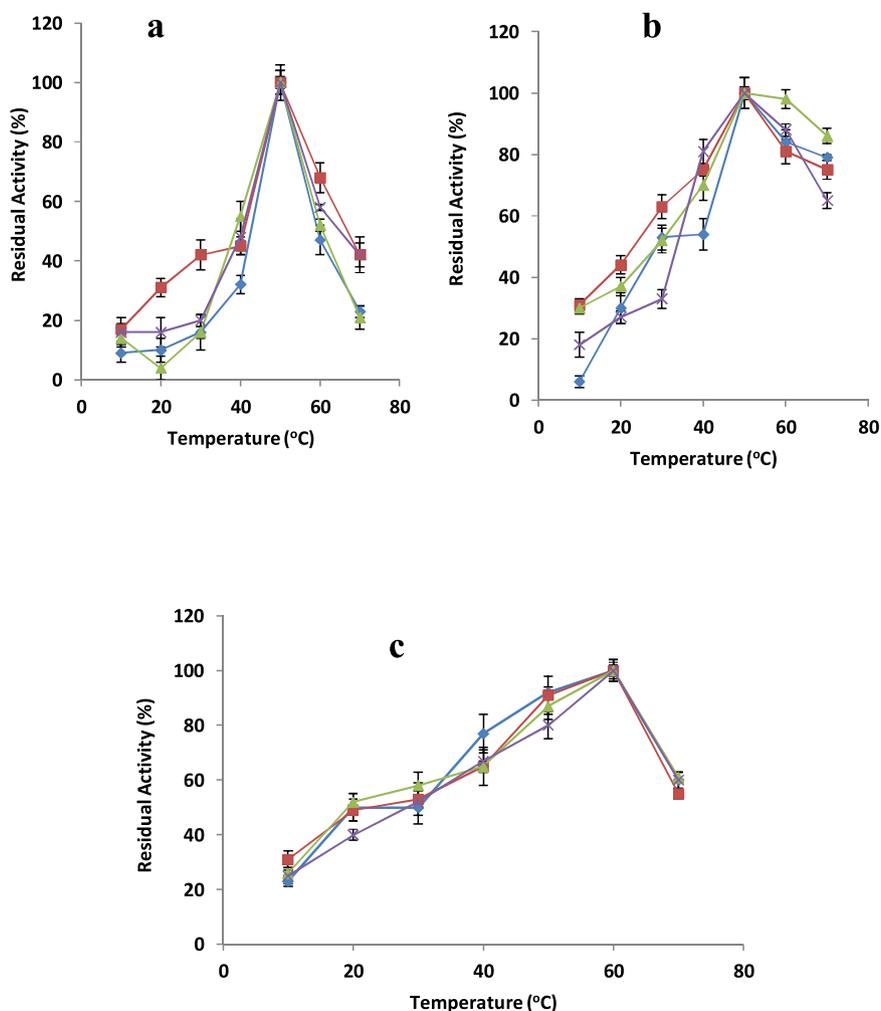
Fig. 5. Effect of temperature on activity of the immobilized *Dioscorea* tyrosinase.
(a) Ca-alginate (b) Polyacrylamide gel (c) CLEAs. The immobilized tyrosinase from *D. praehensilis* (♦), *D. rotundata* (■), *D. alata* (▲) *and C. esculenta* (✕) were incubated in phosphate buffer (50 mM, pH 6.5) at different temperatures and the residual activities were determined under standard assay conditions.

% and 100 % activities at 2nd and 3rd cycles respectively. This could be due to inherent instability of Ca-alginate beads. A decrease in the activity was thereafter noticed at fourth cycle. The rate of activity decline was more pronounced in polyacrylamide gel immobilized enzyme than in other enzyme preparations. At the fourth cycle, the activities retained for the entrapped enzymes in polyacrylamide gel, alginate gel and CLEA were between 30–50 %, 38–52 % and 43–49 % respectively.

3.7. Time course for dopachrome formation by free and immobilized enzyme

Tyrosinase entrapped in CLEA, Ca-alginate and polyacrylamide gel could catalyze formation of dopachrome continuously for a period of 4, 5 and 6 h respectively (Fig. 6). In contrast, free enzymes reached maximum level of dopachrome formation at 2 h.

4. Discussion

Tyrosinase has been a subject of intense investigation in the past two decades because of its increasing use in various biotechnological applications. Our previous work established the presence of tyrosinase in yam tubers high enough to replace the traditional source [30]. In this current study, advances in biotechnology and increasing demand for the enzyme in biotechnology necessitate the

Table 3
Reuseability of immobilized tyrosinase from different yam species.

Cycle No	Relative activity (%)											
	Ca-alginate				Polyacrylamide Gel				CLEA			
	Dp	Dr	Da	Ce	Dp	Dr	Da	Ce	Dp	Dr	Da	Ce
1	100	100	100	100	100	100	100	100	100	100	100	100
2	113	72	84	69	50	70	44	70	85	84	82	74
3	100	59	69	41	50	55	44	55	64	62	68	57
4	50	52	51	38	36	50	32	30	49	47	46	43

The immobilized enzymes were incubated in the L-DOPA substrate and the formation of dopachrome was monitored at 10 min intervals for 1 h. The experiment was repeated for several cycles at 2 h intervals. After each cycle, the immobilized enzyme was washed with 50 mM phosphate buffer, pH 6.5, dried and stored on ice. The activities for subsequent cycles were compared to the first cycle which was taken to be 100 %. *D. praehensilis* (*Dp*), *D. rotundata* (*Dr*), *D. alata* (*Da*) and *C. esculenta* (*Ce*).

development of a shortened purification scheme and methods that will allow the enzymes to be used continuously.

Aqueous two-phase separation (ATPS) employed here as the primary purification step was found to be viable, rapid and efficient since both purification and concentration of the crude enzymes (to about 60 %) were achieved. The extraction conditions resulted in good yield (between 85–124%) and a purification fold (about 4–6 folds) for all the yam tyrosinases. This could be due to partitioning
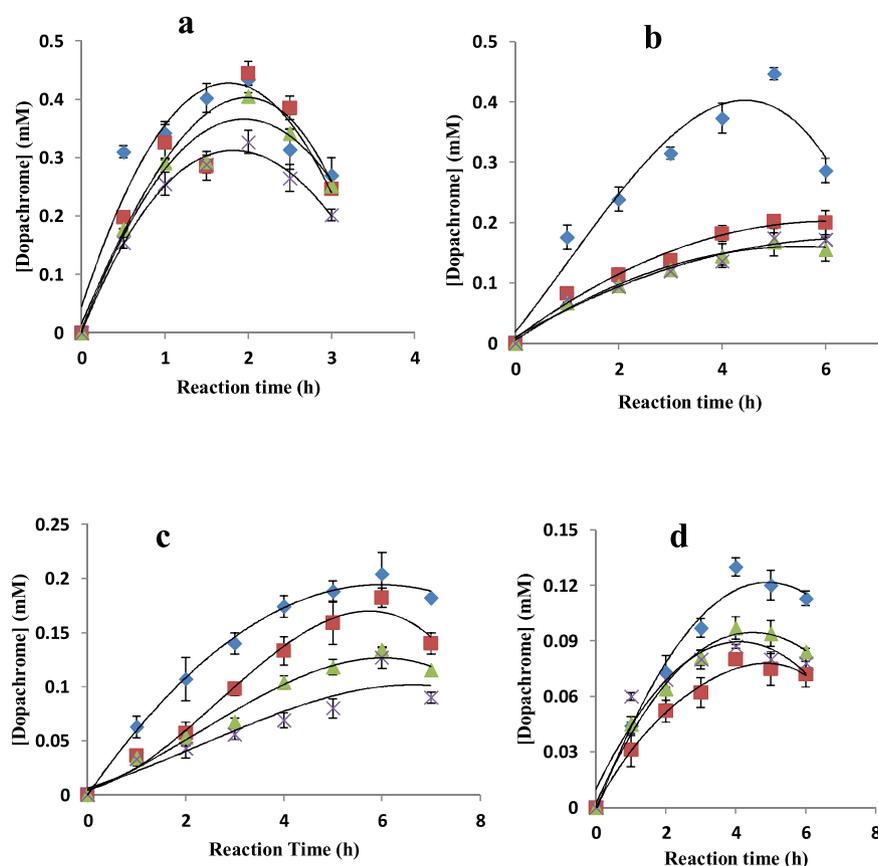
Fig. 6. Time course of dopachrome formation using free and immobilized *Dioscorea* tyrosinase. (a) free enzyme (b) Ca-alginate beads (c) polyacrylamide gel and (d) CLEAs. Both the free and immobilized tyrosinase from *D. praehensilis* (♦), *D. rotundata* (■), *D. alata* (▲) *and C. esculenta* (✕) were incubated in substrate solution containing 1 mM L-DOPA dissolved in 50 mM Na phosphate buffer, pH 6.5 at room temperature. The formation of dopachrome was monitored spectrophotometrically at 1 h interval for a period of 7 h.

of non-target proteins and other contaminants from the desired enzyme to the other phase. Aqueous two phase partitioning was capable of removing 70–80 % of protein impurities. Depending on the type of biotechnological application, this purification step may be sufficient. Gel filtration (size-exclusion) chromatography using Sephadex G-100 was efficient in removing by-products, and other proteins giving a total recovery of between 54–59% and a final purification fold of between 9 and 10 for all the yam sources.

In addition, to ascertain the integrity and apparent purity of the free enzymes, they were subjected to SDS-PAGE. The molecular weights obtained for tyrosinase from all the yam species on SDS-PAGE was equivalent to 41.4 ± 0.2 kDa and the native molecular weights estimated using size-exclusion chromatography was 60.0 ± 3.0 kDa. This is in contrast to 26 ± 0.2 kDa and 54.0 ± 6.7 kDa reported for the free enzymes on SDS-PAGE and size-exclusion chromatography respectively in a previous report (Ilesanmi et al., 2014). Our interpretation of the disparity in the molecular weights is that each mole of tyrosinase molecule binds a mole of polyethylene glycol present in the ATPS, thus increasing the molecular weight from ~54 kDa to the current value of 60 kDa. Probably, the binding of the polyethylene glycol could be such that it intercalated the two subunits probably between ε-NH$_2$ groups of lysine in each of the two subunits, thus preventing the dissociation into monomeric units under denaturing conditions of SDS-PAGE. Pending further experimental investigation, this altered shape and charge could then account for the molecular weight of 41 kDa obtained on SDS-PAGE, since the amount of bound SDS would be reduced thus reducing the mobility on the gel. Modification of serine protease by pegylation,

leading to increase molecular weight and higher catalytic efficiency of the enzyme was reported by da-Silva-Freitas et al. [35]. Radi et al. [36] has reported this type of behavior for lysozyme modification with epoxy-methoxy polyethylene glycol resulting in increased molecular masses of 30–34 kDa. Gooding et al. [37] has also reported that there may be discrepancy in molecular weight as a result of modification of tyrosinase during purification as a result of association with other compounds. Thus, our new purification scheme resulted in a pegylated tyrosinase necessitating further characterization.

Both the turnover (k_{cat}) and the first-order rate constant (k_{cat}/K_m) of the pegylated tyrosinase increased by between 2–4 folds when compared to that obtained earlier by Ilesanmi et al. [30]. This may be partly due to steric rearrangement at the active site of each of the enzyme such that it became more accessible to the substrate as a result of the pegylation. There is also possibility of increased buoyancy of the enzyme due to presence of PEG leading to increased structural dynamics. Similar effect was observed for chemically-modified trypsin with increased k_{cat}/K_m value for the catalysis of catechol [38]. This improved catalytic activity is quite interesting as quite a number of workers have reported decrease in enzyme activity for some chemically-modified enzymes. The reduction of α-chymotrypsin activity after chemical glycosylation with dextran and lactose with a molecular weight of 10 kDa was reported by Solá and Griebenow [39]. Milla *et al.* [40] reported pegylation of proteins and liposomes as a strategy for improving the drug delivery. Decrease in activity of mushroom tyrosinase upon modification with N-bromosuccinimide has also been reported by Emami and Gheibi [41].

Catalysis in organic solvent has been in vogue especially in the area of biotechnology and biocatalysis because when enzymes are placed in organic solvents, they tend to exhibit high selectivity and specificity, increased solubility of substrates and possibility of reduced microbial contamination. The new tyrosinase preparations obtained in this study showed superior stability in organic solvents when compared to those purified using conventional methods. This may be probably due to pegylation of the enzyme as such has been reported to increase dispersion of protein in solvents because of its amphiphilic nature [42]. Enzyme immobilization is a very important aspect of biotechnology as it offers many advantages over the soluble enzymes for several industrial processes. The binding interactions including ionic, hydrophobic and other secondary interactions between the enzyme and the alginate or change in the enzyme microenvironment could be responsible for the shift in pH for maximal activity. Such observation was reported by Palmieri et al. [33] on fungal phenol oxidase immobilized on Cu-alginate gel. Munjal and Shawney [43] also reported similar observation on mushroom tyrosinase immobilized on gelatin gel. Optimum pH of 7.0 was reported for tyrosinase immobilized on polyacrylonitrile beads [44].

Optimum temperature for the polyacrylamide and Ca-alginate entrapped tyrosinase preparations was 50 °C which was the same as that of free enzyme. In contrast, tyrosinase CLEA had a shift in optimum temperature to 60 °C. This could be due to rigid CLEA structure caused by the cross-linking which may require higher temperature to attain proper structural conformation for catalysis. Increase in optimum temperature and thermal stability for CLEA have also been reported in the work of Aytar and Bakir [45] and Xu et al. [28]. The inert nature of acrylamide may be responsible for tyrosinase entrapped in polyacrylamide gel having the same optimum pH and temperature with that of the free enzyme.

The immobilized enzyme was used for four different repeated cycles and their activities diminished upon repeated uses. The decrease in activity and progressive blackening of the immobilized enzyme after repeated use might be as a result of accumulation of polymeric products from the remaining substrates trapped within the gel. These polymeric products might have clogged the pores thereby causing diffusional obstacle in the accessibility of the substrate to the enzymes active site. Although, similar observation was reported in the work of Harir et al. [46], this problem is currently being addressed.

5. Conclusion

A shortened purification scheme for yam tyrosinase has been established together with the possibility of immobilizing the enzyme on different supports. The several interesting properties of the free and immobilized enzyme preparations may be of interest in many applications.

Authors' statement

All the authors agreed and jointly made the revisions suggested on the manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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