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Research article

Potentials of purified tyrosinase from yam (*Dioscorea* spp) as a biocatalyst in the synthesis of cross-linked protein networks

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ABSTRACT

We report the usefulness of yam tyrosinase as a catalyst in the synthesis of cross-linked protein networks for biopolymers. The enzyme was purified using aqueous two-phase partitioning (ATPs) and peptide mapping on SDS-PAGE was carried out to ascertain degree of similarities of tyrosinase from the yam species. The mapping revealed distinct peptide bands of 3, 4, 4 and 2 for tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* respectively purified using conventional method. In contrast, continuous broad band was noticed for the ATPS-purified enzymes due to bound polyethylene glycol (PEG). Tyrosinase from *D. praehensilis* with overall better properties was used in the synthesis of cross-linked protein networks. The enzyme catalyzed conversion of soluble proteins from whey, moringa leaves, pumpkin leaves and cow blood into fibrous (cross-linked) protein networks for improved properties and functionalities. The purified tyrosinase from *D. praehensilis* was also covalently bonded to bovine serum albumin (BSA) forming tyrosinase-BSA adduct with molecular weight of 118 ± 2.0 kDa, revealing its potential as a reporter enzyme by reporting BSA. The overall result further reinforces yam tyrosinase as an enzyme of interest in various biotechnological applications.

1. Introduction

Tyrosinases are copper enzyme that catalyzes the oxidation of phenolic compounds to their quinone derivatives, further converted to melanin, a ubiquitous pigment in living organisms (Zekiri et al., 2014; Salah Maamoun et al., 2021). They engaged in hydroxylation of monophenolic substrates to *o*-diphenols and further conversion of diphenols to *o*-quinones with concomitant reduction of molecular oxygen to water (Ba and Kumar, 2017). Tyrosinases are distributed ubiquitously in all organisms (Halaouli et al., 2006). They are found in micro-organisms, plants and animals. Tyrosinase is the most thoroughly studied polyphenol oxidase because of its participation in biosynthesis of melanin and skin pigmentation (Yu and Chang, 2004), as well as undesired browning reactions in fruits and vegetables (Seo et al., 2003). They play a role in the regulation of the redox potential of respiration in cell and wound healing activities in plants (Mayer, 2006). Browning reactions which occur when mechanical injuries are inflicted on some plant tissues like tubers, fruits and vegetables during processing or post-harvest operations have been associated with tyrosinase. This has been studied in banana (Wuyts et al., 2006), walnut leaves (Zekiri et al., 2014), loquat fruit (Zhang and Shao, 2015). The enzymatic browning reactions are initiated by endogenous tyrosinases, which oxidise the phenolic compounds present in the tissues when their cells are broken (Zolghadri et al., 2019). These browning reactions cause changes in food products' organoleptic properties and appearance, leading to a short shelf-life and a lower market value. Initial studies of tyrosinase were motivated by a desire to understand and to prevent the enzymatic browning that occurs when plants, fruits or vegetables are cut or when mechanical injuries are inflicted on them. The phenomenon was ultimately linked to the action of tyrosinase resulting into severe economic losses in the food industry. This necessitated several studies on the tyrosinase inhibitors. The research focus has moved to the biotechnological and environmental applications of the enzyme. Due to their ability of reactions with phenolic substrates, the enzymes have been proposed for use in various biocatalysis and biotechnological applications (Nawaz et al., 2017); such as in detoxification of contaminant soils and phenol-containing waste water (Martorell et al., 2012), as additives in food processes (Selinheimo et al., 2007); conjugation of protein gelatin to polysaccharide chitosan (Chen et al., 2002); tailoring polymers (Anghileri et al., 2007), synthesis of organic compounds such as L-3, 4- dihydroxyphenylalanine (L-DOPA), used in the treatment of Parkinson's disease (Ates et al., 2007) and in synthesis of cross-linked protein networks (Tian et al., 2019).

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Tyrosinase has continued to attract the attention of biotechnologists and new uses are developed as such, the development of a rapid purification method for the enzyme to meet the industrial and biotechnological demand is necessary. Conventional purification are laborious and time-consuming (Srinivas et al., 1999). Aqueous two-phase partitioning method of purification employed here is fast, cost-effective and viable. It also offers low energy consumption, biocompatible environment to the enzyme and combines both purification and concentration of the resulting enzyme molecule (Srinivas et al., 1999). Purified enzymes with improved activity and stability could easily be manipulated for use in several biotechnological processes such as synthesis of organic compound, ability to form cross-linked protein networks etc. Enzymatic cross-linking of proteins has gained increasing interest in food technology to create novel food products or improve textural properties of dairy products and biopolymers (Ahmed et al., 2019).

The covalent assembly of proteins into macromolecular networks by enzyme catalysis has been extensively investigated in various areas of applications where biological protein matrices with material-like structural and mechanical properties are required. The ability of enzymes to form cross-linked protein networks has, for instance, been exploited to modify the texture and appearance of food products, to develop new biomimetic tissue scaffolds, or to strengthen protein-based fibers for textile fabrication. Permana et al. (2020) reported polymerization of protein by sterically controlled enzymatic crosslinking. Also, the effect of tyrosinase-catalyzed crosslinking on the structure and allergenicity of turbot parvalbumin mediated by caffeic acid has been reported (Tian et al., 2019).

In our first work, Ilesanmi et al. (2014), the presence of tyrosinase in four yam species (*Dioscorea praehensilis*, *Dioscorea alata*, *Dioscorea rotundata* and *Colocasia esculenta*) was investigated and reported. Kinetic characteristics of the purified enzymes in aqueous and non-aqueous media were also reported. In further work, a fast and cost-effective method of purification for the yam tyrosinase was developed, in addition to possibility of immobilizing the enzyme on different supports (Ilesanmi and Adewale, 2020).

In this present work, we focused on application of the enzyme as a catalyst in the synthesis of cross-linked protein networks. We have chosen whey, cow blood, moringa and pumpkin leaves as sources of soluble proteins. These proteins are underutilized and/or always discarded. Whey and cow blood with relatively high amount of protein are always thrown away. Also, moringa and pumpkin leaves are good sources of plant proteins more especially to vegetarians, but mastication of the leaves to obtain proteins may not be appropriate. Hence, the reason for enzymatic conversion to a fibrous insoluble networks (crosslinks) for improved functionalities, that could further be exploited in food and dairy industries.

2. Materials and methods

2.1. Materials

Four different yam tubers-*Dioscorea praehensilis*, *Dioscorea rotundata*, *Dioscorea alata*, and *Colocasia esculenta*, were obtained from farms around Ile-Ife environs, southwestern Nigeria. The yam cultivars were authenticated at the IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife.

2.2. Preparation of yam homogenate and tyrosinase activity determination

Homogenates from the four (4) yam species were prepared according to the method reported by Ilesanmi and Adewale (2020). The supernatants were assayed for tyrosinase activity using L-3, 4-dihydroxyphenyalanine (L-DOPA) as substrate and stored at -20 °C, when not used immediately.

Tyrosinase activities with L-3, 4-dihydroxyphenylalanine (L-DOPA) were determined according to the method of Lerch and Etlinger (1972) as modified by Ilesanmi et al. (2014).

One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μmol of product (*o*-dopaquinone) per minute at 475 nm under the specific assay condition ($\varepsilon_{475} = 3600\ M^{-1}\ cm^{-1}$).

$$\text{Activity }(\mu\text{mole/min}) = \frac{\Delta OD475nm \times V \times DF}{\varepsilon \times v}$$

where V = total assay volume (ml), DF = dilution factor, ε = Extinction coefficient of product (3600 $M^{-1}cm^{-1}$) and v = volume of enzyme used for assay. Specific activity which represents the activity of an enzyme per milligram of total protein, was obtained from total activity divided by total protein. It is expressed as μmol min^{-1} mg^{-1}.

2.3. Determination of protein concentration

The protein concentrations in the crude homogenates and purified tyrosinase preparations were determined as described by Bradford (1976) using BSA as the standard protein.

2.4. Enzyme purification

The enzymes were purified using two methods (Method A and B). Method A involved a combination of ion-exchange using CM-Sepharose (cation exchanger) and QAE-Sephadex A-50 (anion exchanger) and gel filtration chromatography. Active pools from both cation and anion exchangers were further purified using gel filtration on Sephadex G-100 (Ilesanmi et al., 2014). Method B involved combination of aqueous two-phase partitioning (ATPS) and gel filtration chromatography. Post ATPS pools were purified further on Sephadex G-100 as reported by Ilesanmi and Adewale (2020).

2.5. Peptide mapping of tyrosinase preparations

Peptide mappings of tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* were carried out according to the method of Cleveland et al. (1977). Appropriate volume of each of the enzyme equivalent to 30 μg protein, was dissolved in a buffer containing 0.125 M Tris/HCl at pH 6.8, 0.5% SDS, 10% glycerol, and 0.0001% bromophenol blue. The sample mixtures were then heated to 100 °C for 2 min. Digestion was carried out by addition of chymotrypsin to achieve 133 μg/ml in the mixture. The mixture was incubated at 37 °C for 30 min. 2-mercaptoethanol and SDS were added to a final concentration of 10% and 2% respectively. The digestion was stopped by boiling the samples for 2 min and loaded after cooling, into the sample well of 17% acrylamide gel. Electrophoresis was carried out according to the method described by Laemmli (1970). . After electrophoresis, the protein bands were stained overnight in 1% Coomassie brilliant blue R-250 solution and destaining was done in a solution containing 10% acetic acid and 10% methanol in distilled water.

2.6. Synthesis of fibrous protein networks

Extraction of proteins from whey, cow blood, moringa and pumpkin leaves was carried out following the standard procedure. The moringa and pumpkin leaves were homogenized in 50 mM phosphate buffer, pH 7.0 containing 150 mM NaCl and centrifuged at 5500×*g* for 20 min to extract the proteins into the supernatants. The supernatants were left on ice for 24 h to precipitate out the chlorophyll from the soluble proteins. The cow blood sample was collected in a beaker containing 3% trisodium citrate as anticoagulant. The blood sample was centrifuged at 2000×*g* for 10 min at 4 °C using cold centrifuge. The resulting supernatant (plasma) was then separated from the red blood cells into clean plastic screw-cap vials. The protein concentration in all the samples (whey, cow blood, moringa and pumpkin leaves) was thereafter determined according to Bradford (1976) using BSA as standard. The synthesis of fibrous protein network using soluble proteins (which are otherwise discarded) from

Table 1. Purification summary of tyrosinase purified using aqueous two-phase partitioning.

Sample	Steps	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)	% Yield	Purification fold
D. praehensilis	Crude	166680.0	92.0	1812 ± 0.2	100.0	1.0
	ATPS	180550.0	20.0	9028 ± 0.3	108.0	5.0
	SEC	91650.0	6.0	15275 ± 0.2	55.0	9.0
D. alata	Crude	156960.0	12.0	13080 ± 0.7	100.0	1.0
	ATPS	134176.0	2.6	52320 ± 0.1	86.0	4.0
	SEC	93296.0	0.8	112488 ± 0.0	59.0	9.0
D. rotundata	Crude	161120.0	92.0	1751 ± 0.5	100.0	1.0
	ATPS	200010.0	25.0	8000 ± 0.2	124.0	5.0
	SEC	94815.0	6.0	15759 ± 0.2	59.0	9.0
C. esculenta	Crude	48080.0	164.0	293 ± 0.2	100.0	1.0
	ATPS	52728.0	30.0	1758 ± 0.2	110.0	6.0
	SEC	28197.0	9.4	3000 ± 0.0	54.0	10.0

SEC – Size-exclusion Chromatography. ATPS – Aqueous two phase partitioning. The data are the mean ± standard deviation (s.d.) of three independent determinations.

whey, cow blood, moringa and pumpkin leaves with purified tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* were carried out following the method of Wu et al. (2013). The reaction mixture for the synthesis contained appropriate amount of the protein samples, 1 mM caffeic acid, and tyrosinase. The mixture was incubated at 40 °C for 4 h. In control samples, tyrosinase solution was substituted with distilled water. The products were observed with a Zeiss LSM 510 META confocal microscope fitted to a Zeiss Axiovert 200 M.

2.7. Covalent coupling of dioscorea tyrosinase with BSA

The potentials of purified tyrosinase as a reporter enzyme was investigated by coupling the enzyme with BSA according to the method of Ayhan et al. (2012) with modifications. This involved mixing BSA with the enzyme in a 1:1 M ratio. The mixture was subjected to stirring and 1% glutaraldehyde was added. The resulting solution was stirred for 15 min and incubated at room temperature for 4 h. The reaction was terminated by separation of the mixture on Sephacryl S-300 column. The protein profile of the fractions were measured at 280 nm and fractions were assayed for tyrosinase activity.

2.8. Statistical analyses

All experiments were repeated three times and the data reported as mean ± standard deviation. Other statistical analysis was performed with GraphPad Prism 5, version 5.01.

3. Results and discussion

3.1. Enzyme purification

The purification process (ATPS) resulted into high yield of 80% or more and a purification fold of between 4 to 6 folds for all the enzyme (Table 1). This may be due to phase partitioning of the enzyme from other proteins and contaminants. On size exclusion chromatography, total recovery of ≥54% and final purification fold of between 9 and 10 for the yam sources were obtained. The molecular weight of the ATPS-purified tyrosinase were observed to be 61 kDa in contrast to 55 kDa expected and obtained for those purified conventionally. This was confirmed on SDS-PAGE. The subunit molecular weight obtained on SDS-PAGE was 41 kDa as compared to 27 kDa for the enzyme purified conventionally. It was observed that the tyrosinase probably became pegylated after purification by ATPS. This observation was further confirmed by peptide mapping.

3.2. Digestion and detection of peptide maps of tyrosinase preparations

Figure 1 shows the peptide patterns of purified tyrosinase upon digestion with chymotrypsin. The number of distinct peptide bands observed for the purified tyrosinase (using method A) from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* were 3, 4, 4 and 2 as shown in B, C, D and E respectively (Figure 1a). However, for the tyrosinase preparations using method B, a continuous broad band was noticed for all the enzymes (Figure 1b). Peptide mapping has been a

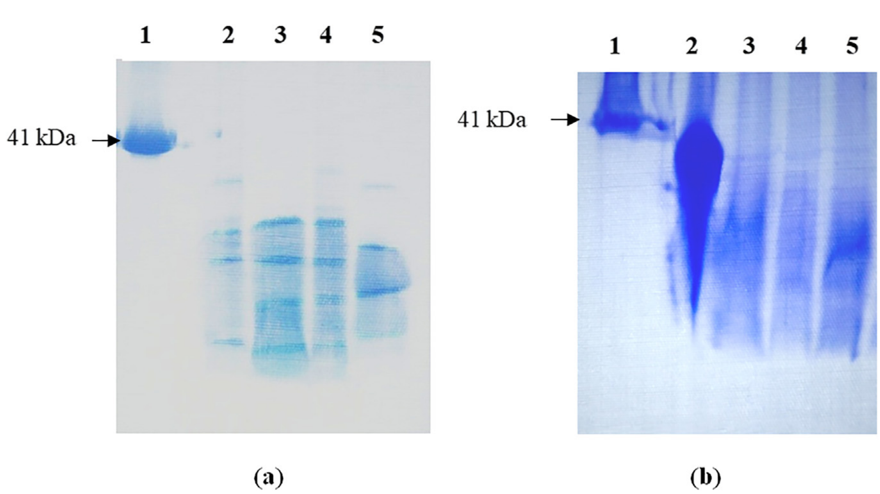

Figure 1. Peptide patterns of pegylated and unpegylated tyrosinase digested with chymotrypsin. (a) For the unpegylated enzymes-Lane 1 represents undigested tyrosinase while 2, 3, 4 and 5 represent digested tyrosinase from *D. praehensilis*, *D. alata*, *D. rotundata* and *C. esculenta* respectively. The digestion was carried out with 133 μg/ml of chymotrypsin and incubated at 37 °C for 30 min. (b) For the pegylated enzymes-Lane 1 represents undigested tyrosinase while 2, 3, 4 and 5 represent digested tyrosinase from *C. esculenta*, *D. rotundata*, *D. alata*, and *D. praehensilis* respectively.

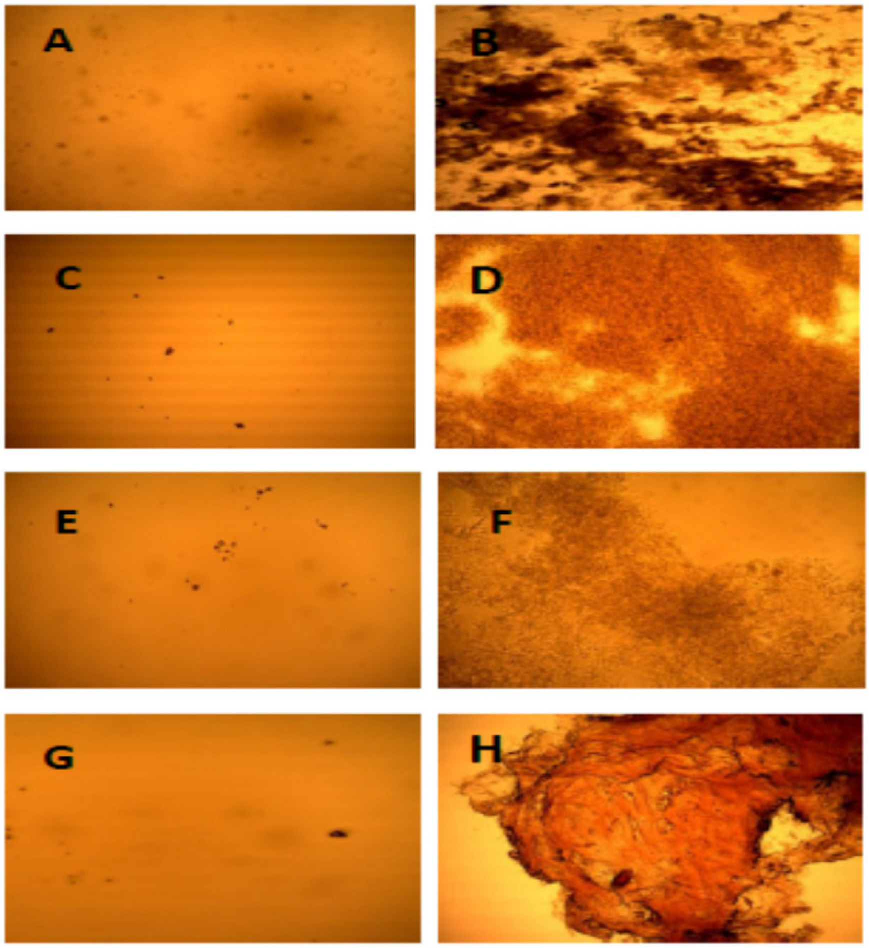

Figure 2. Photomicrographs of Cross-linked Protein Networks. The synthesis of fibrous protein networks were carried out under the following reaction conditions: caffeic acid (1 mM), tyrosinase (900 U/ml) were incubated at 40 °C for 4 h. A: Whey protein + incubation mixture without tyrosinase while B: whey protein after incubation with 1 mM caffeic acid + tyrosinase. C: Pumpkin protein + incubation mixture without tyrosinase while D: pumpkin protein after incubation with 1 mM caffeic acid + tyrosinase. E: Moringa protein + incubation mixture without tyrosinase while F: moringa protein after incubation with 1 mM caffeic acid + tyrosinase. G: Cow blood protein + incubation mixture without tyrosinase while H: cow blood protein after incubation with 1 mM caffeic acid + tyrosinase.

useful method in proteomics to characterize primary structure of proteins by selective cleavage to yield a predictable set of peptides. We have designed peptide mapping as a comparative procedure to provide further information on disparities in the Mr of tyrosinase obtained using different purification methods. The peptide bands of method A tyrosinase preparation after chymotryptic digestion and electrophoresis were quite distinct (Figure 1a). The differences in their pattern revealed that tyrosinase from different species of yam are not identical proteins. However, in the preparations obtained using method B, the chymotrypsin was probably trapped by the PEG molecule on the surface of the protein forming PEG-chymotrypsin adduct which restricted the digestion, resulting in broad bands with no clear separation (Figure 1b). This provided additional information that the enzyme was probably pegylated in the course of purification with method B.

3.3. Synthesis of fibrous protein network

The estimated protein concentration in whey, cow blood, moringa and pumpkin leaves were 5.0 ± 0.6, 51.1 ± 21, 0.6 ± 0.1 and 21.2 ± 3.7 mg/ml respectively. The polymeric fibrous structure obtained after the

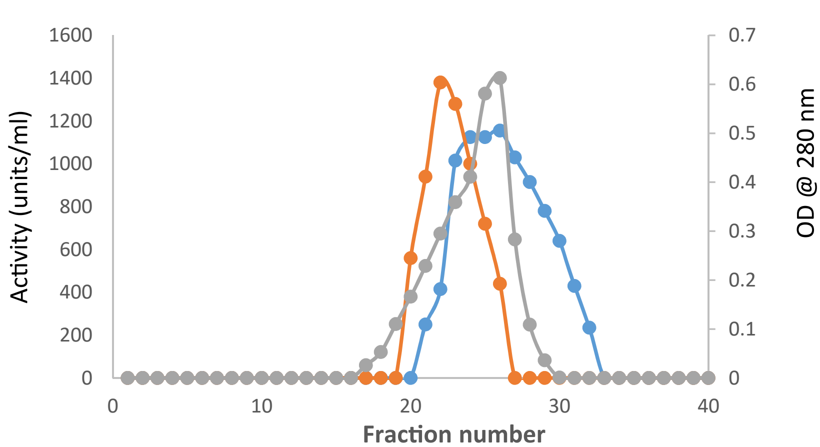

Figure 3. Elution Profiles of Tyrosinase, BSA and Tyrosinase-BSA Conjugate on Sephacryl S-300 column. Tyrosinase, BSA and tyrosinase-BSA conjugate were applied separately to a column of Sephacryl S-300 previously equilibrated with 50 mM phosphate buffer, pH 6.5. The flow rate was 12 ml/h. Fractions of 1 ml each were collected for each of the samples and were analyzed. A single protein and activity peak equivalent to a molecular weight of 66.9 kDa each was obtained for BSA and tyrosinase respectively while the single activity peak obtained for the tyrosinase-BSA conjugate had a molecular weight of 118.6 kDa. This is an indication that the tyrosinase has been cross-linked with BSA without loss of tyrosinase activity.

cross-links is shown in Figure 2. No network was formed in the absence of tyrosinase. Enzymatic cross-linking of proteins has gained increasing interest in food technology to create novel food products or improve textural properties of dairy products and biopolymers (Ahmed et al., 2019). Permana et al. (2020) reported polymerization of protein by sterically controlled enzymatic crosslinking. Also, the effect of tyrosinase-catalyzed crosslinking on the structure and allergenicity of turbot parvalbumin mediated by caffeic acid was reported by Tian et al. (2019).

Cross-linking of proteins has been exploited in modification of solubility, foaming and emulsifying properties of food products. Based on the cross-linking, a new functional three-dimensional protein networks are created (Thalmann and Lotzbeyer, 2002). Transglutaminase has been the traditional enzyme in the synthesis of cross-linked protein networks (Bonisch et al., 2007). The use of yam tyrosinase in the effective cross-linking of proteins was achieved in this study. The enzyme could catalyze the formation of fibrous protein networks from soluble proteins obtained from whey, cow blood, moringa and pumpkin leaves (Figure 2). It has been shown that crosslink formation between proteins that are not accessible to tyrosinases can be induced by the addition of small-molecule phenolic compounds such as caffeic acid (Fairhead and Thöny-Meyer, 2010). These molecules likely function as crosslinking mediators to overcome the absence of surface-exposed tyrosine residues on the target proteins. The protein networks formed by yam tyrosinase could further be developed to a meat protein system for vegetarians. It could also be converted to thread-like material for application as suture in post-operative stitches.

3.4. *Dioscorea* tyrosinase as reporter enzyme

Figure 3 shows the elution profile of tyrosinase, BSA and tyrosinase-BSA conjugate on calibrated Sephacryl S-300. A single peak equivalent to molecular weight of 66.9 kDa each was obtained for the tyrosinase and BSA respectively. When the tyrosinase was cross-linked with BSA, a single peak with molecular weight of 118.6 kDa was obtained indicating that all the BSA molecules were cross-linked with the tyrosinase forming a single product with tyrosinase activity. Formation of tyrosinase dimer would require other bonds like hydrogen bonds and van Der Waals between non-polar side chains of the enzyme. For tyrosinase to be linked together, it forms cross-linked tyrosinase aggregate (CLEA) and becomes immobilized. Hence, nullifies the possibility that tyrosinase dimer was formed. Tyrosinase makes use of surface exposed tyrosyl residues present in its structure to link together with other molecules (BSA) especially in the presence of agent like glutaraldehyde without becoming insoluble aggregate. In this experiment, the tyrosinase linked together with BSA in presence of the cross linker without becoming immobilized, resulting into higher molecular mass of 118kDa.An ELISA kit has been known to detect antigens, antibodies and proteins by producing an enzyme triggered change of colour (Al-Shaban et al., 2010). Horseradish peroxidase is probably the most common enzyme used as reporter enzymes because of their ability to produce a chromogenic product even at low concentrations. The possibility of using yam tyrosinase as a reporter enzyme has also been established in this work. The enzyme was cross-linked with BSA using glutaraldehyde as a crosslinker showing that the BSA protein has been reported by the tyrosinase activity. This could also be applied to other proteins or enzymes to form a molecule with new properties and activities.

4. Conclusion

A newly devised purification scheme which tends to shorten the purification process time with improvement in the catalytic properties has been established. The purified enzyme could catalyze the cross-linking of soluble proteins to form fibrous protein networks. Its potential has as a reporter enzyme was also established. The overall results further reinforce yam tyrosinase as a future industrial enzyme.

Declarations

Author contribution statement

Olutosin Samuel Ilesanmi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Omowumi Funke Adedugbe: Contributed reagents, materials, analysis tools or data.

Isaac Olusanjo Adewale: Contributed reagents, materials, analysis tools or data; Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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