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Quantitative and Qualitative Detection of Vitamin C in Some Foods by Immobilized Ascorbate Oxidase

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Abstract

The use of ascorbate oxidase immobilized with glass-alginate gel beads led to immobilized 93% of the enzyme original amount, the optimum pH of immobilized enzyme was 5.5, and it was stable at 5-6, but it loses 63% from its original activity at pH 7, while the optimum temperature was 40°C, and it was stable at 50°C for 15min, but it loses more than 85% from its original activity at 60°C for same time, the immobilized ascorbate oxidase retained its full activity for 30 days, but it retained 84.36% of its original activity after storage for 60 days at 4°C, and enzyme retained full activity for 25 continue usage; while it retained 94.29% of its original activity after 30 continue usage. The results for determination of ascorbic acid by ascorbate oxidase immobilized with glass-alginate gel beads in some foods refer to edible parts of fruits and vegetables were content 0.7416, 0.5428, 0.5193, 0.4168, 0.3852, 0.3649, 0.2973, 0.2637, 0.1835, 0.1824 and 0.1472mg/1g in kiwi, strawberry, lemon, orange, carrot, spinach, cabbage, mandarin, green pepper, tomato and radish respectively.

Keywords: Vitamin C; Immobilized Enzymes; Ascorbate Oxidase.

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1. Introduction

Ascorbic acid (vitamin C) is a water-soluble vitamin and the most common electro active biological compound and one of the most ubiquitous vitamins ever discovered which can be found in many biological systems and foodstuffs (fresh vegetables and fruits, namely, citrus) [1]. The chemical formula is (R)-5-((S)-1,2-dihydroxyethyl)-3,4-dihydroxyfuran-2(5H)-one, molar mass 176.12g $mol^{-}1$, density 1.65g/cm^3, melting point 190-192°C, with solubility in water about 33g/100ml [2]. It is essential in the human nutrition, as humans do not have the enzymes necessary to synthesize it although most animals can synthesize vitamin C from D-glucose and the recommended dietary allowances (RDA) have been established for adult women of 75mg/day and 90mg/day for men [1]. Vitamin C is plays an important role in forming collagen, a protein that gives structure to bones, cartilages, muscles, teeth, maintain capillaries, iron absorption in the gut, carnitine biosynthesis, blood vessels, immune response activation, as a reducing agent in the cellular metabolism, cofactor for several enzymes, synthesis of adrenal hormones, metabolism of folic acid, tyrosine and tryptophan and also acts as a powerful antioxidant which fights against free radical induced diseases. [3,4]. Vegetables and fruits considered as rich sources include citrus fruits, green leafy vegetables, broccoli, cauliflower, blackcurrant, tomatoes, peppers, and potatoes, are major food sources of vitamin C [1,3]. Several techniques were used for the determination of ascorbic acid, which includes spectrometry, titration, the electrochemical, and enzyme based potentiometric. Among all above methods the potentiometric based enzyme technique has many advantages including low cost, highly selective, sensitive, and more accurate and giving very quick response to the analytic. [5]. Enzymatic method is considered a commonly used method which depend of L-ascorbate oxidase (EC 1.10.3.3) that belongs to the family of oxidoreductases, who is responsible of produced de hydro ascorbic acid as a product of L-ascorbic acid, so the product of enzyme will provide a signal for detection of ascorbic acid quantity [6]. Enzyme immobilization is confinement of enzyme to a phase (matrix/support) different from the one for substrates and products. Inert polymers and inorganic materials are usually used as carrier matrices. Apart from being affordable, an ideal matrix must encompass characteristics like inertness, physical strength, stability, regenerability, ability to increase enzyme specificity/activity and reduce product inhibition, nonspecific adsorption and microbial contamination [7,8], So, Various approaches have been used for ascorbate oxidase immobilization for determination of L-ascorbic acid either covalently or non covalently by different methods on polymer matrices such as ZnO Nanorods [5], nylon net through glutaraldehyde covalent bond [9], poly(3,4-ethylenedioxythiophene)/ multi walled carbon nanotubes composite films [10], nanostructured TiO_2 films [11], gelatin zinc oxide nano composite [12], poly(3,4-ethylenedioxythiophene) and multi walled carbon nanotubes composite films [13], nylon (Biodyne A) membrane by glutaraldehyde [14] and glass pearls glutaraldehyde [15]. So, this study amid to immobilization of ascorbate oxidase and study some characterization and using to determination of vitamin C in some foods.

2. Materials and methods

2.1. Place of research

All experiments on this Research were conducted in the laboratories of the market research and consumer protection center, university of Baghdad, Iraq in 2015.

2.2. Preparation of samples

Samples of vegetables and fruits were cut into small parts, and 10gm of these parts were homogenized with 50ml of 1M phosphate buffer solution pH 5.5 in a blender, the crude solution was centrifugation for 20min at 5000rpm, the supernatant was used to estimate of ascorbic acid [6].

2.3. Enzyme immobilization

Glass beads (Next advance GB05, 0.5 mm) were activated by soaking for 1.5h in a 10% glutaraldehyde solution prepared in deionized water, after that washed by cooled deionized water on Whatman paper No. 1, then 0.2gm of activated support was putted in 5ml of 1M phosphate buffer solution pH 5.5 and add 1ml of ascorbate oxidase 10mg/ml (Sigma) and leave it under stirring at 4°C for 24h, then washing with 100ml of cooled same buffer was use to removed non immobilized enzyme and then with 500ml of 1M NaCl to any non covalent bonds from support [15]. Then sodium alginate solution 2.5% (w/v) and glass beads were mixed in the ratio of 2:1 (v/v). The mixture was passed drop wise into 2% (w/v) $CaCl_2$ solution. The formed beads were retained in the stirred $CaCl_2$ solution (using a magnetic stirrer) at least for 2h for gel hardening. The ascorbate oxidase-glass-alginate beads had 3-4 mm in diameter. Finally, the beads were separated and washed with deionized water 3 times. Before using, the beads were immersed in 1M phosphate buffer solution pH 5.5 [16].

2.4. Estimated of enzyme activity

Immobilized ascorbate oxidase activity were estimated by mixed 10ml of enzyme-glass-alginate beads with 20mL of 1M phosphate buffer solution pH 5.5 containing 1ml of 3mM L-ascorbic acid (or sample solution), the mixture was kept with stirring at 25°C and 200rpm for 5min. Control was run without L-ascorbic acid. The enzyme activity was expressed as the decrease in absorbance at 256mn [17]. Enzyme unit was defined as one unit of ascorbate oxidase with oxidized 1µM of ascorbic acid per one min [6].

2.5. Determination of protein concentration

Protein concentration (mg/ml) was determined according (Bradford, 1976) [18] using bovine serum albumin as a standard protein at 595nm.

2.6. Enzyme Loading

The enzyme loading was calculated by measuring the difference between protein concentration (mg/ml) of enzyme solution that add to activated support (glass beads-glutaraldehyde) (At_0) and same solution after stirring activated support with at 4°C for 24h (At_t). The immobilization yield (IY) was calculated with the following equation (Adriano et al., 2005)[19]:

$$IY(\%) = \frac{At_0 - At_t}{At_0} \times 100$$

2.7. Optimization of experimental conditions

Immobilized ascorbate oxidase was optimized by used same experimental conditions for determination of enzyme activity with several changes as the following (Liu et al., 2011b)[13].

2.8. pH

At 4, 4.5, 5, 5.5, 6, 6.5 and 7.

2.9. Temperature

At 25, 30, 35, 40, 45, 50, 55 and 60°C.

2.10. Storage stability

For 60 days at 4°C.

2.11. Number of use

Follow up enzyme activity after each use up to 30 times.

2.12. Standard vitamin C (ascorbic acid) solution

0.1g standard crystalline ascorbic acid was dissolved in 1000ml distilled water to prepare 100µg\ml standard stock solution.

3. Results and discussion

3.1. Enzyme loading

The use of ascorbate oxidase immobilized with glass-alginate gel beads led to immobilized 93% of the enzyme original amount (Figure 1).

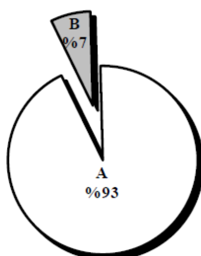

Figure 1: Ascorbate oxidase loading of glass-alginate gel beads, when:

A: The percentage of enzyme (%) which immobilized with glass-alginate gel beads from total amount of the

enzyme (mg / ml).

B: The percentage of enzyme (%) that had not immobilized with glass-alginate gel beads from total amount of the enzyme (mg / ml).

3.2. Optimum pH

The optimum pH of ascorbate oxidase immobilized with glass-alginate gel beads was 5.5, and it was stable at 5-6, but it loses 63% from its original activity at pH 7 (Figure 2).

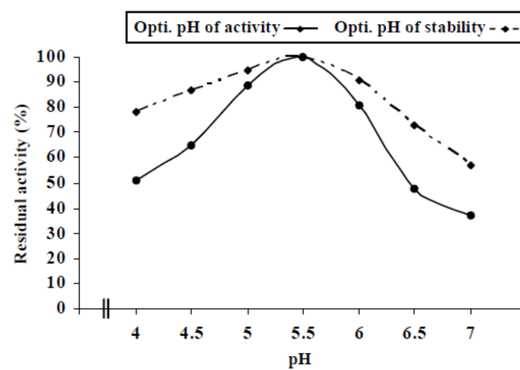

Figure 2: Optimum pH of the activity and stability of ascorbate oxidase immobilized with glass-alginate gel beads

3.3. Optimum temperature

The optimum temperature of immobilized with glass-alginate gel beads was 40°C, and it was stable at 50°C for 15min, but it loses more than 85% from its original activity at 60°C for same time (Figure 3).

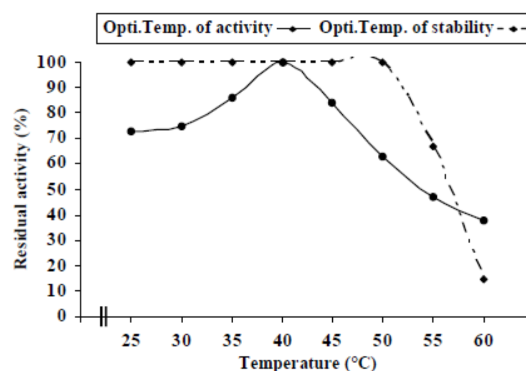

Figure 3: Optimum temperature of the activity and stability of ascorbate oxidase immobilized with glass-alginate gel beads.

3.4. Storage stability

The immobilized ascorbate oxidase with glass-alginate gel beads retained its full activity for 30 days, but it

retained 84.36% of its original activity after storage for 60 days at 4°C (Figure 4).

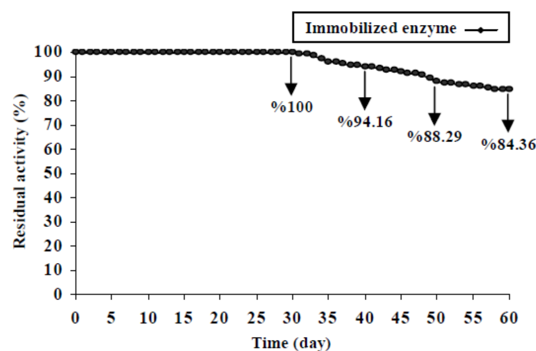

Figure 4: Storage stability of ascorbate oxidase immobilized with glass-alginate gel beads.

3.5. Number of usage times

The immobilized ascorbate oxidase with glass-alginate gel beads retained its full activity for 25 continue usage; while it retained 94.29% of its original activity after 30 continue usage (Figure 4).

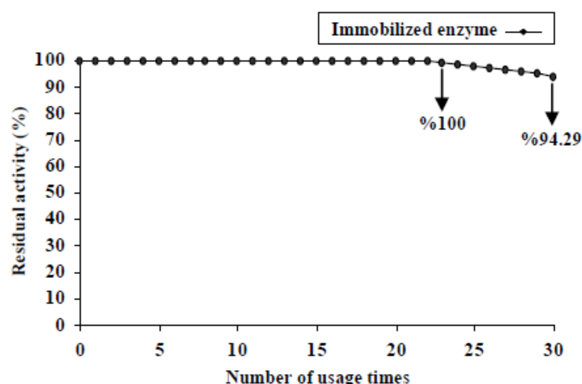

Figure 5: Number of usage times of ascorbate oxidase immobilized with glass-alginate gel beads.

3.6. Determination of ascorbic acid in some foods

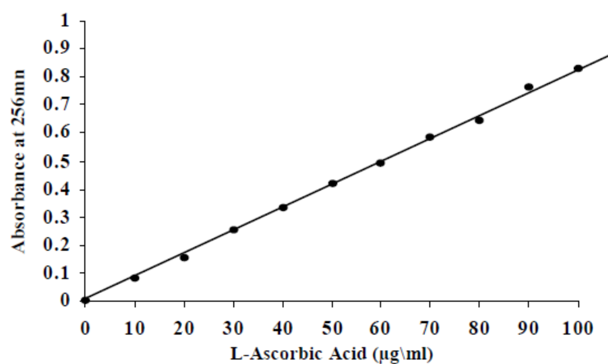

Figure 6: Standard curve of L-ascorbic acid concentration 10-100 µg/ml.

Standard curve of L-ascorbic acid solutions were assayed with the immobilized ascorbate oxidase showed a linear relationship between ascorbate oxidase activity and L-ascorbic acid concentration (10-100 µg/ml) (Figure 6).

The results (Table 1) refer to content of vitamin C in some edible parts of fruits and vegetables by ascorbate oxidase immobilized with glass-alginate gel beads, it was 0.7416, 0.5428, 0.5193, 0.4168, 0.3852, 0.3649, 0.2973, 0.2637, 0.1835, 0.1824 and 0.1472mg/1g in kiwi, strawberry, lemon, orange, carrot, spinach, cabbage, mandarin, green pepper, tomato and radish respectively.

Table 1: Total content of vitamin C in some edible parts of fruits and vegetables by ascorbate oxidase immobilized with glass-alginate gel beads.

Fruits and vegetables	Vitamin C (mg/1g)
Kiwi	0.7416
Strawberry	0.5428
Lemon	0.5193
Orange	0.4168
Carrot	0.3852
Spinach	0.3649
Cabbage	0.2973
Mandarin	0.2637
Green pepper	0.1835
Tomato	0.1824
Radish	0.1472

Results obtained in this study was comparable with some other studies, Total content of vitamin C was 0.470, 0.358, 0.643, 0.107 mg/ml in Orange, Grapefruit, Strawberry, Tomato respectively when it determined by immobilized ascorbate oxidase with Ca-alginate gel beads (Esaka et al 1985). While it was 50, 60, 75, 4, 34.3, 33.1 and 12.3 mg/100g in lemon, strawberry, kiwi, carrot, cabbage, spinach and radish respectively when it determined by Shimada and Ko. (2008). Also, it was 4.337, 5.315, 2.808, 51.74, 0.841, 17.416 and 1.557 mg/10g in orange, lemon, mandarin, strawberry, tomato, cabbage and green pepper respectively (Mohammed et al 2009). Moreover, immobilized ascorbate oxidase onto pore glass was used for detection of ascorbic acid in natural juices (lemon, orange and kiwi) (Danet et al 2000). The immobilized enzyme with nylon (Biodyne A) membrane by glutaraldehyde was used for ascorbic acid content assessment in several real samples such as soft drinks, natural commercial fruit juices, as well as natural juices freshly prepared by fruit squeezing (Pisoschi et al **2010).**

4. Discussion

4.1. Enzyme loading

It is noted that the degree of immobilized obtained was largely encouraging for adoption in this study, also, this result was similar with (Esaka et al., 1985)[17] that found only 9% of the native enzyme activity was retained after the immobilization with Ca-alginate gel beads.

Immobilization process was need to use some materials which are often a inert polymer and inorganic materials as well as it available at non expensive prices, as it should be characterized by important properties such as high strength, stability toward interaction materials, ability to increase or save of enzyme activity witch immobilized it by not effects on recognize, binding and catalysis location in active site or not deterring on the binding conformation between enzyme and substrate for the completion of the reaction and produce the required products, as well as its ability to immobilized highest amount of the enzyme [7].

4.2. Optimum pH

The pH optimum of the activity and stability for enzyme was considered one of the important parameters that define immobilization success, because the change value of pH for immobilized enzyme lead to inability to use in specific areas, thus the feasibility of immobilized enzyme to become dependence on use of free enzyme, because it gives a greater economic importance. So, link between enzyme and immobilized material (glass-alginate gel beads) did not lead to any change in optimum pH of activity, the reason of that due to that this link did not happen in important location such as recognize, binding and catalysis in active site that it's responsible of enzyme activity, as well as that this link did not affect on the enzyme conformation when link with substrate to do its work correctly interaction [8]. Immobilized enzyme with Ca-alginate gel beads, showed higher activities at pH 6 [7]. The optimum activity of immobilized enzyme onto glass pearls and glutaraldehyde was pH at 5.8-6 [15]. Maximum activity of immobilized enzyme onto nylon net through glutaraldehyde covalent bond at pH 5.8 [9]. Ascorbate oxidase immobilized ZnO nanorods biosensor gradually increases up to pH 5 and after that it tends to decrease because of the less stability of L-ascorbic acid in neutral or alkaline solution [5]. The optimum activity occurred at around pH 6.2 for immobilized enzyme with poly(3,4-ethylenedioxythiophene) and multi walled carbon nanotubes composite films [13].

4.3. Optimum temperature

The increase of temperature leads to decrease in enzyme activity as a result of thermal denaturing of enzyme molecule because the high temperature working on open folds of enzyme molecule and exposure of content from amino acids to the reaction medium, thus, it affected of high temperatures [8]. So, many studies were referring to this parameter. The maximum temperature response of ascorbate oxidase immobilized on ZnO nanorods biosensor was 55°C [5]. Although the biosensor of immobilized enzyme with poly(3,4-ethylenedioxythiophene) and multi walled carbon nanotubes composite films showed a maximum response at about 40°C, room temperature 25°C was still chosen to keep the stability of the biosensor and to prevent possible solution evaporation at higher temperature [13]. While, immobilize ascorbate oxidase onto nanostructure TiO_2 films were found to be thermally stable up to 40°C [11].

4.4. Storage stability

The storage stability is an extremely important parameter for a immobilized enzymes, so, many research were refer to this parameter. The reactor with immobilized ascorbate oxidase with glass pearls and glutaraldehyde was under for 3 weeks without any loss in enzymatic activity, and was reduced about 55% of its initial activity

[15]. Immobilized enzyme with nylon (Biodyne A) membrane by glutaraldehyde was constant for 10 days at 4°C [14]. Ascorbate oxidase immobilized ZnO nanorods biosensor retained about 90% of its original activity for three weeks stored at 4°C [5]. While, immobilized enzyme onto poly(3,4-ethylenedioxythiophene) and multi walled carbon nanotubes composite films remained at 99% of its initial activity for the first 10 days, and 65% activity was retained after one month [13]. Whilst, immobilize ascorbate oxidase onto nano structured TiO_2 films have a shelf life of about 4 weeks when stored at 4°C [11]. Moreover, enzyme that immobilized with gelatin zinc oxide nano composite retained about 88% of activity even after 8 weeks of preparation when stored under refrigerated conditions at 4°C [12].

4.5. Number of usage times

The number of usage times for immobilized enzymes reuse was represents one of the main economic factors when thinking about immobilization because it gives a clear conception about the efficiency of materials which are used for this purpose. The enzyme thus immobilized with Ca-alginate gel beads lost no activity at all even when the enzyme reaction was repeated over 50 times [17]. No significant loss in enzymatic activity of immobilized enzyme onto nylon net through glutaraldehyde covalent bond was observed when carried out using it almost daily during 25 days leading to 500 injections [9]. At the end of the 24th experiment for AA, the biosensor of immobilized enzyme onto poly(3,4-ethylenedioxythiophene) and multi walled carbon nanotubes composite films retained 97% of its original activity [13].

4.6. Determination of ascorbic acid in some foods

Results obtained in this study was comparable with some other studies, Total content of vitamin C was 0.470, 0.358, 0.643, 0.107 mg/ml in Orange, Grapefruit, Strawberry, Tomato respectively when it determined by immobilized ascorbate oxidase with Ca-alginate gel beads [17]. While it was 50, 60, 75, 4, 34.3, 33.1 and 12.3 mg/100g in lemon, strawberry, kiwi, carrot, cabbage, spinach and radish respectively when it determined by (Shimada and Ko, 2008)[6]. Also, it was 4.337, 5.315, 2.808, 51.74, 0.841, 17.416 and 1.557 mg/10g in orange, lemon, mandarin, strawberry, tomato, cabbage and green pepper respectively [20]. Moreover, immobilized ascorbate oxidase onto pore glass was used for detection of ascorbic acid in natural juices (lemon, orange and kiwi) [15]. The immobilized enzyme with nylon (Biodyne A) membrane by glutaraldehyde was used for ascorbic acid content assessment in several real samples such as soft drinks, natural commercial fruit juices, as well as natural juices freshly prepared by fruit squeezing [14].

5. Conclusion

Immobilized ascorbate oxidase with glass-alginate gel beads provides an economical and reliable method for the determination of L-ascorbic acid in foods with high speed and accuracy.

6. Limitations of the study

This study was used one materials (glass-alginate gel beads) to immobilized of ascorbate oxidase and study some characteristics of immobilized enzyme like (amount of lodging, optimum pH and temperature, storage

stability and number of usage times) after that the application of immobilized enzyme was determination of ascorbic acid in some foods (fruits and vegetables).

7. Recommendation

Study of others materials to immobilized enzyme by different methods in immobilization to determine the best material and method that lead to get the best results.

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