

Optimization of Various Factors Affecting Glucose Oxidase Activity Produced by *Aspergillus niger*

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ABSTRACT

The enzyme glucose oxidase was produced by fermentation technology, using *Aspergillus niger* as fermentation organism and glucose as substrate. The highest specific activity of glucose oxidase and enzyme recovery was found to be 35.54 Umg⁻¹ protein and 97.5% respectively, in the fractions precipitated with 80% ammonium sulphate saturation. Another partial purification by dialysis resulted in raising the specific activity of glucose oxidase to 37.44 Umg⁻¹ proteins with purification folds 2.16 times. Further purification of the enzyme by gel filtration technique on sephadex G200 Column at pH 5.6 increased the specific activity of the enzyme to 65.2 Umg⁻¹ proteins with 3.76 purifications fold. The maximum activity of the purified glucose oxidase *A. niger* was reached on using 0.25% of glucose as a substrate in the enzymatic reaction mixture and incubating at 37°C for two hours at PH 5.6. Amino acid Composition of pure glucose oxidase was characterized by containing 13 amino acids, among which serine, glycine, glutamic acid, aspartic acid and alanine constitutes (more than 60% of total amino acids).

Key Words: Glucose oxidase; Production; *Aspergillus niger*; Culture optimization

INTRODUCTION

Enzymes can be defined as soluble colloidal organic catalysts which are produced by living cells and are capable of acting independently of the cells (Rao, 1993). Glucose oxidase belongs to oxidoreductase and is also called as glucose aerodehydrogenase (Witteveen *et al.*, 1990). Glucose oxidase (β -D-glucose: oxygen-1-oxidoreductase, EC 1.1.3.4) is a fungal enzyme which catalyses the oxidation of β -D-glucose (C₆H₁₂O₆) to gluconolactone (C₆H₁₀O₆) and the concomittant reduction of molecular oxygen in hydrogen peroxide (H₂O₂). The gluconolactone then hydrolyzes spontaneously to gluconic acid (C₆H₁₂O₇).

The glucose oxidase from fungal origin especially, Ascomycetes is generally glycoprotein with molecular weight about 155000 and consisting of two identical polypeptide chain subunits linked by disulfide bonds, optimum pH for the enzyme action is 5.5 with a broad range from 4 to 7 (Bentley, 1959). Glucose oxidase appears mostly to be stable and can be stored for long time (Rando *et al.*, 1997). Production of extracellular glucose oxidase (GO) by *Aspergillus niger* during its growth on glucose and non glucose carbon sources was investigated (Rinas *et al.*, 2001). It was possible to use molasses as a carbon source and enhancing the enzyme activity approximately 40 folds, indicating an economically attractive process for enzyme production (Hatzinikolaou & Macris, 1995). Glucose oxidase is produced by many microorganisms such as *Penicillium notatum*, *Penicillium chrysosporium*, *Aspergillus niger* and *Botrytis cinerea* (Lium *et al.*, 1998; Hafiz *et al.*, 2003).

Glucose oxidase from *Aspergillus niger* GO is an homodimeric secreted protein intracellular enzyme present

in the mycelium of the organism (Willis, 1966 cited by Hafiz *et al.*, 2003). For purification of fungal glucose oxidase enzyme, particularly from *Aspergilli* and *Penicillia*, a simple extraction step of the mycelial mass, followed by cation exchange chromatography and gel filtration were elaborated, which is competitive with those of the commercially produced enzymes (Kim *et al.*, 1990; Rando *et al.*, 1997). The high substrate affinity and specificity makes glucose oxidase a suitable biocatalyst for industrial applications (Danneel *et al.*, 1993). The amino acid composition of glucose oxidases from fungal sources was investigated by several workers (Nakamura & Fujiki, 1989; Kim *et al.*, 1990).

Glucose oxidase is of considerable commercial importance due to its high specificity for glucose, its high turnover and its high stability. Therefore, it has a wide range of applications (Kona *et al.*, 2001). In industry, it is used to produce gluconic acid and as a source of hydrogen peroxide in food preservation. In diagnostics, it is the basis of sensors for the estimation of glucose concentration in blood, serum or plasma. The aim of the present work is to investigate the optimization and production of glucose oxidase from *Aspergillus niger* in ultimate use for special application in biotechnology and industries.

MATERIALS AND METHODS

Microorganism and fermentation. Pure cultures of *Aspergillus niger* were isolated from cultivated Egyptian soil samples as well as waste materials of sugar cane processing collected from Hawamedia Distilleries factories.

It was grown on potato starch agar slants pH 4, sporulation medium. It was incubated aerobically at 30°C

for 72 h. Conical flasks each containing 100 mL medium pH 4.0 with different concentrations of micro nutrients were inoculated with 10 mL of homogenous spore suspension, and incubated at 30°C on a shaker (120 rpm) for the optimum fermentation period (Zubair *et al.*, 2002). The fermented biomass in each case was filtered and then blended to extract intracellular enzyme, and the filtrates were centrifuged at 15000 rpm for 15 min in a cooling centrifuge (Kim *et al.*, 1990). The supernatant was through filter paper to separate the mycelium pellet from the culture filtrate. The clear supernatant considered as the crude enzyme was assayed for glucose oxidase (Zia, 2002).

Optimization of enzyme activity. The reaction mixture (D-glucose) as a substrate was fermented with *Aspergillus niger* oxidase enzyme at different concentrations varying from (0.05 – 0.5 µg mL⁻¹), temperatures from (10°-60°C), and pH from (2-8) of the reaction mixture (Shalaby, 1999). Also, pH stability of the enzyme activity was tested at a range of pH values of 2-8 in shake flask. The experiments were carried out in such a way that the parameter optimized in one experiment was maintained in the subsequent investigation (Monreal & Reese, 1969).

Enzyme assay. Glucose oxidase activity in the crude enzyme extract was determined by spectrophotometer method at 460 nm wavelength using glucose as a substrate and o-dianisidine buffer as coupling reagent (Worthington, 1988). The assay is based on the estimation of residual reducing sugar was carried out using the method of (Monreal & Reese, 1969). While the amount of reducing sugar released in the supernatant was determined by (Ressig *et al.*, 1955; Shindia *et al.*, 2001) using N-acetyl glucosamine as standard.

Estimation of protein. The protein content of the crude enzyme preparation was determined by folin-phenol reagent according to the method of Lowery *et al.* (1951) and Ohnishi and Barra (1978), using bovine serum albumin as a standard.

Purification of intracellular glucose oxidase. The crude enzyme preparation was subjected to different purification steps as shown in Table I. All purification steps were carried out at 4°C as follows:

Step 1. Ammonium sulphate fractionation. (Dixon & Webb, 1964). The precipitation of crude intracellular glucose oxidase enzyme preparation was carried out by adding different amounts of ammonium sulphate to give saturation from (20 to 100%). The solution was left overnight at 4°C until the complete precipitation occurred, and then centrifuged at 15000 rpm for 15 minutes to remove the undissolved particles. Each fraction precipitate was dissolved immediately in a known volume (20 mL) of 0.1 citrate phosphate buffer (pH 5.6). The dissolved fractional precipitates were tested for both glucose oxidase activity and protein content.

Step II. Dialysis. This step of purification was carried out to remove the traces of ammonium sulphate in the final fractional precipitates. The precipitate enzyme preparation

obtained from 80% ammonium sulphate fraction which was introduced inside a dialysis bag (Cellulose bag) and dialyzed overnight against pure sucrose crystals (Ammar, 1975). The concentrated partially purified glucose oxidase preparation was diluted by the same citrate buffer to known volume (50mL) at 4°C. The glucose oxidase activity and protein content were also estimated.

Step III. Gel filtration. A column of sephadex G200 was used for purifying the concentrated dialyzed enzyme preparation (for gel filtration 40-120 µ fractionation range from 1000 to 200.000 MW) as mentioned by Ammar (1975) and Shindia *et al.* (2001). The sephadex G200 column (2.6 × 70.0 cm) has been used. This sephadex was swollen in 0.1 M citrate phosphate buffer, pH 5.6 and eluted again with the same buffer at a flow rate of 20 mL h⁻¹ at room temperature. Fractions of 5 mL were collected and stored at (-20°C) until use. The glucose oxidase activity and protein content were examined for all different fractions separately.

Amino acids Analysis. Amino acid composition of the partial purified glucose oxidase (Sharp peak fractions obtained from gel filtration G200) was determined according to the methods of Kim *et al.* (1990) with modification by Sherif (1998). Amino acid determination was performed at Central Laboratory for Food and Feed (CLFF), Agriculture Research Center, Ministry of Agriculture, Giza, Egypt, applying the GLC hydrolytic analysis technique, with a Beckman amino acid analyzer system 7300. A dialyzed sample of partial purified enzyme (1 mg) and hydrolyzed in a sealed ampoule with 2 mL of constantly boiling 6 NH dL containing 0.75 µl of 2 mercapto ethanol was added. The ampoules were sealed under vacuum and then heated in an oven at 110°C for 24 hrs. then opened and the excess of HCl was removed from the hydrolysates in vacuo and suspended in 2 mL of citrate phosphate buffer (pH 2.2). Half mL aliquots were injected in Beckman amino acid analyzer for analysis.

Statistical analysis. The data were statistically analyzed as a complete randomized block design. Means is compared by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The crude enzyme preparation of *A. niger* was subjected to purification using the conventional protein purification methods including precipitation and gel filtrations techniques (Table I).

Fractional precipitation of *A. niger* glucose oxidase. Crude glucose oxidase enzyme produced under optimum conditions by *A. niger* was subjected to fractional precipitation with ammonium sulphate (Gomori, 1955). The results presented in Table II clearly show that the specific activity, recovery and purification fold in the precipitates reached their maximal values at 80% ammonium sulphate concentration giving 35.54 units mg⁻¹ protein with enzyme recovery of 97.5% and 2.05 fold indicated that 80% ammonium sulphate saturation was optimum for glucose oxidase precipitation as shown in Fig. 1. The partially

Table I. A summary of purification and overall recovery of glucose oxidase produced by *A. niger*

Purification steps	Volume (mL)	Total glucose oxidase activity (U/100 mL)	Total protein content (mg/mL)	Specific activity (U/mg protein)	Purification folds	Yield %
Control	100	102.1	5.90	17.31	1	100
Ammonium sulphate 80% saturation	100	99.5	2.80	35.54	2.05	97.5
Dialysis by sucrose	10	5.00	2.68	37.44	2.16	97.9
Sephadex G ₂₀₀	5	4.20	0.07	65.203	3.76	41.1
Mean ±SD	43.00±52.12	42.16±53.57	2.29±2.43	31.10±24.39	2.24±1.14	52.85±53.06

U: The amount of enzyme that required to convert 1.0 ug of D-glucose per half hour at 37°C

Table II. A summary of precipitation pattern of *A. niger* glucose oxidase by ammonium sulphate

Ammonium Sulphate conc. %	Volume (mL)	Total glucose oxidase activity (U/100 mL)	Total protein content (mg/100 mL)	Specific activity (U/mg protein)	Enzyme Yield %	Purification Fold
Control *	100	102.1	5.9	17.31	100	1
20	100	37.1	1.8	20.61	36	1.19
40	100	58.4	2.3	25.38	57.2	1.47
60	100	78.6	2.6	30.21	77.1	1.57
70	100	86.7	2.7	32.93	84.9	1.75
80	100	99.5	2.8	35.54	97.5	2.05
100	100	99.0	3.2	30.9	77.0	0.97
Mean ±SD		80.20±24.44	3.04±1.33	27.55±6.69	75.67±22.63	1.43±0.40

*Control; Determination was carried out without precipitation with ammonium sulphate fractionation.

purified precipitate from 80% saturation ammonium sulphate was dissolved in 0.1 M citrate phosphate buffer (pH 5.6). The dialysis step (Table I) increased the specific activity to 37.44 U/mg protein and the purification folds to 2.16 folds with an overall yield of 97.9%. The dialysed enzyme preparation was applied to the top of a sephadex G200 column and subjected to gel filtration yielding 30 fractions as shown in Fig 2. Glucose oxidase activity was detected only in fraction numbers from 30–40, after which no activity could be recovered. Fraction number 33 was the most active containing the highest specific activity of glucose oxidase activity (65.20 U/mg proteins) showing 3.76 fold purification. These results indicate that *A. niger* intracellular glucose oxidase is composed of one enzyme component. This observation is in line with that previously reported by (El-Enshasy, 2003; Liu *et al.*, 2001; Kona *et al.*, 2001), which indicated that glucose oxidase from *A.niger* is an intracellular enzyme associated with the mycelium. Many investigators have used ammonium sulphate and organic solvent for the precipitation of the intracellular glucose oxidase enzyme and other microbial enzymes (Rando *et al.*, 1997; Sherif, 1998; Tohamy & Shindia, 2001).

The amino acid composition of *A. niger* glucose oxidase enzyme (Sharp Peak fractions, 1 mg) was characterized by being rich in serine, glycine, glutamic acid, aspartic acid and alanine which represented the most abundant residues in *A. niger* glucose oxidase i.e. more than 60% of total amino acids. The results in Table III indicated that 13 amino acids (Table IV) were detected in the enzyme sample. However, isoleucine was least abundant residue in enzyme. It is interesting to mention that the amino acids, proline, cysteine, methionine as well as arginine were not determined in glucose oxidase from the tested local fungus. The results of the present work were in partial agreement with other fungal glucose oxidase enzymes particularly

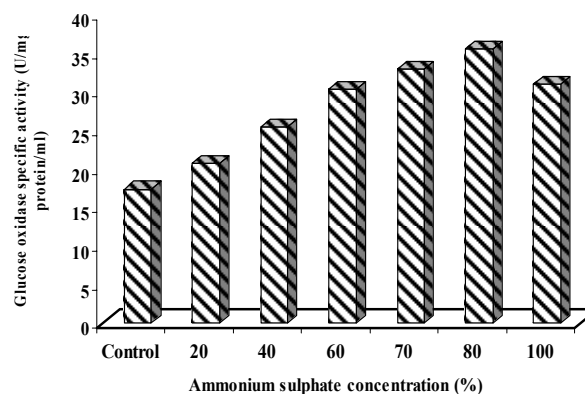
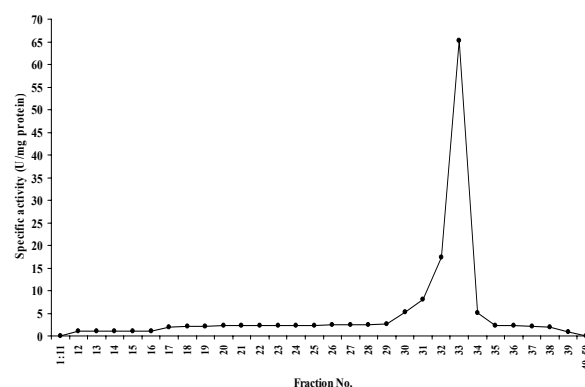
Fig. 1. Relation of the applied ammonium sulphate conc. to the corresponding precipitated activity of glucose oxidase of *A. niger* Control: Determination was carried out without precipitation with ammonium sulphate fractionation**Fig. 2. Gel filtration of partially purified *A. niger* glucose oxidase on Sephadex G200 Column chromatography at pH 5.6.**

Table III. Fractionation pattern of (GO) produced by *A. niger* local isolate on sephadex G-200 column chromatography

Fraction No.	(GO) (U/mL)	Protein content (mg/mL)	Specific activity U/mg protein/mL
1-11	0	0	0
12	0.004	0.004	1.050
13	0.005	0.005	1.062
14	0.009	0.008	1.065
15	0.013	0.012	1.071
16	0.027	0.025	1.092
17	0.062	0.031	2.014
18	0.089	0.043	2.082
19	0.091	0.043	2.112
20	0.114	0.052	2.193
21	0.158	0.071	2.222
22	0.175	0.077	2.264
23	0.188	0.082	2.292
24	0.205	0.088	2.323
25	0.214	0.091	2.350
26	0.226	0.095	2.384
27	0.250	0.100	2.448
28	0.261	0.105	2.489
29	0.275	0.108	2.548
30	0.606	0.115	5.267
31	0.968	0.119	8.134
32	2.110	0.121	17.440
33	4.243	0.119	65.203
34	0.540	0.105	5.147
35	0.245	0.109	2.248
36	0.062	0.028	2.222
37	0.044	0.021	2.107
38	0.036	0.018	1.986
39	0.022	0.024	00.91
40-50	0.00	0.000	0.00

Table IV. Amino Acid Composition of GO produced by *A. niger* (Mole %)

Amino acid	Mole (%) of amino acid in glucose oxidase
Asparagines (ASP)	10.39
Threonine (THR)	4.55
Serine (SER)	16.28
Glutamic (GLU)	12.37
Proline (PRO)	ND
Glycine (GLY)	14.84
Alanine (ALA)	8.05
Cysteine (CYS)	ND
Valine (VAL)	3.71
Methionine (MET)	ND
Isoleucine (ILE)	3.30
Leucine (LEU)	5.77
Tyrosine (TYR)	3.71
Phenylalanine (PHE)	5.56
Histidine (HIS)	4.74
Lysine (LYS)	6.19
Arginine (ARG)	ND

ND= Not determined

from *Aspergillus* and *Penicillium* species (Kim *et al.*, 1990).**Effect of PH.** In this experiment, the optimum pH for

glucose oxidase activity produced from *A. niger* was determined at different pHs ranging from 2 to 8 using citrate phosphate buffer (0.1 M of citric acid). As shown in Table V, the results showed maximum activity of glucose oxidase (65.2 U/mg^{-1} proteins) at pH 5.6 and 5.0. The specific activity of the enzyme was gradually decreased below or above these pH optima to reach a minimum value at pH 2.0 showing about 20.6% less than the optimal activity. Thus, a loss of about 69% in enzyme activity was recorded than that of maximal activity obtained. The results of Rando *et al.* (1997) are in good agreement with our results in case of glucose oxidase of *Penicillium pinophilum*. They determined that the optimum pH for glucose oxidase production ranged between pH 4.0 to 6.0 reaching 50% activity at pH 3.0 and 7.5. The pH stability of *A. niger* glucose oxidase was also estimated by incubating the purified enzyme for suitable period (2 hrs) at 37°C at various pHs values ranging from 2.0 to 8.0 using citrate phosphate buffer. There after enzyme reaction mixture readjusted to pH 5.0 and enzyme assayed in the normal manner. The data in Fig. 3 revealed that enzyme stability followed the pH activity curve rather closely. The purified enzyme activity was stable at pH ranging from 4.0 to 6.0. The pH stability of the enzyme under investigation was comparable to those obtained for other fungal glucose oxidases (Kim *et al.*, 1990, Rando *et al.*, 1997).

Effect of temperature. Glucose oxidase optimum temperature of activity was 37°C (Table VI, Fig. 4). Further increase of the temperature up to 66°C resulted in marked loss in enzyme activity. This is in accordance with the findings of (Kim *et al.*, 1990; Rando *et al.*, 1997) who reported that 30–40°C were the optimum temperatures for *Talaromyces flavus* and *P. pinophilum* glucose oxidase activities.

Effect of substrate level. The maximum activity of glucose oxidase 65.20 U/mg^{-1} proteins was observed with 0.25% glucose. It is clear from the results in Table VII and Fig. 5 that lower or higher glucose concentration than 0.25% gave lesser enzyme activity due to saturation or to product inhibition. These observations are in line with those of (Kim *et al.*, 1990; Rando *et al.*, 1997) which indicated that, glucose oxidase are highly specific for D-glucose substrate and they stated that, D-glucose is oxidized in the presence of molecular oxygen at the C-position to D-gluconic acid.

CONCLUSIONS

From these results obtained by the production of glucose oxidase and protein content measured mainly in cell-free extracts, our conclusions can be summarized as follows:

Table V. Effect of PH-value of the reaction mixture on glucose oxidase activity of *A. niger*

PH – value	Specific activity (U/mg protein)	Relative activity (%)
2	13.43	20.60
3	30.30	46.50
4	49.26	75.50
5	65.20	100.00
Control (5.6)	65.20	100.00
6	65.00	99.69
7	32.67	50.11
8	20.14	30.89
Mean ± SD	42.65±21.29	70.34±31.89

Table VI. Effect of reaction temperature on glucose oxidase activity of *A. niger*

Temperature °C	Specific activity (U/mg protein)	Relative activity (%)
10	25.16	38.59
20	45.11	69.19
30	64.80	99.38
37	65.20	100.00
40	60.11	92.19
45	42.10	64.69
50	20.11	30.84
60	0.00	0.00
Mean ± SD	40.32±23.60	61.86±36.20

Table VII. Effect of substrate D-glucose concentration in reaction mixture on intracellular glucose oxidase activity of *A. niger*

Glucose concentration µg/mL	Specific activity (U/mg protein)	Relative activity (%)
0.05	30.11	46.18
0.1	45.50	69.78
0.2	55.60	85.53
0.25 control	65.20	100.00
0.3	65.10	99.84
0.4	63.18	96.90
0.5	62.12	95.27
Mean ± SD	55.26±13.12	84.79±20.13

- Partial purification of the crude intracellular glucose oxidase produced by *A. niger* was carried out by fractional precipitation at 80 % ammonium sulphate correspondent the highest specific activity and recovery of that enzyme, while with the following partial purification by mean of the dialysis with pure sucrose crystals the specific activity relatively increased up to 37.44 U/mg protein with purification folds 2.16 times.
- Further purification of the partially purified enzyme was achieved using the gel filtration technique on Sephadex G-200 column at pH 5.6. The increase in the specific activity of glucose oxidase enzyme is significantly enhanced till 65.20 U/mg protein with purification folds 3.76 times.
- The pure glucose oxidase of *A. niger* was characterized regarding glucose substrate concentration, temperature as well as pH optima. The maximum activity of the purified glucose oxidase of that *A. niger* was found on

Fig. 3. Effect of pH-value on the stability of glucose oxidase activity U/mg protein of *A. niger* at 37°C per half hour.

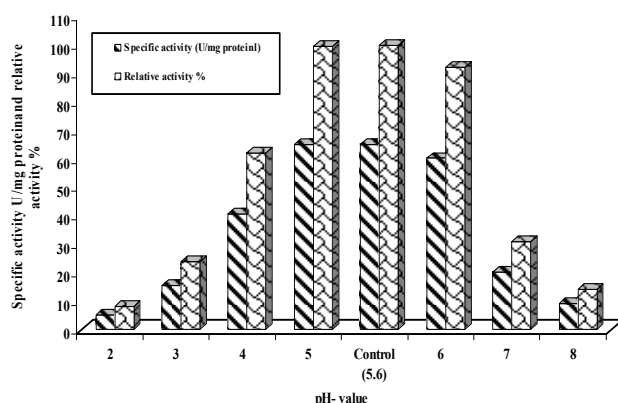


Fig. 4. Effect of reaction temperature on glucose oxidase activity of *A. niger*

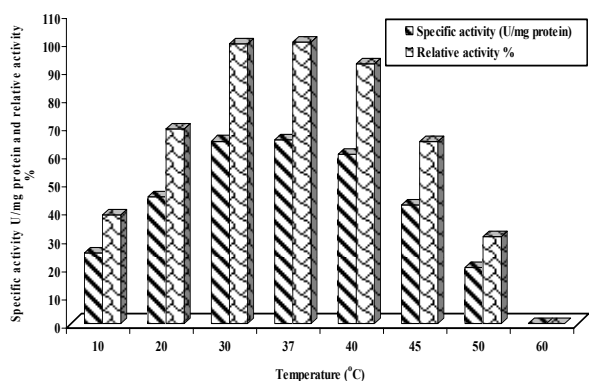
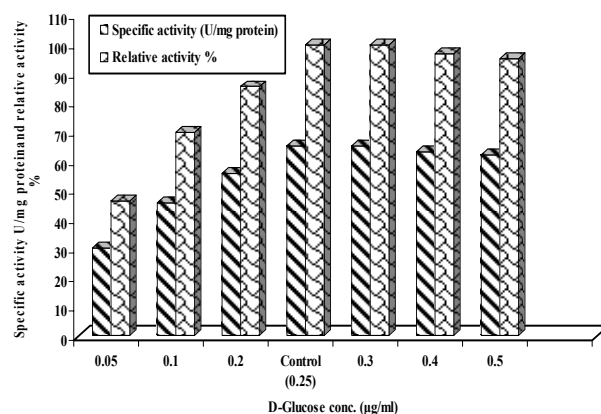


Fig. 5. Effect D-Glucose concentration in reaction mixture on intracellular glucose oxidase activity of *A. niger* at 37°C per half hour.



using 0.25 % of glucose substrate in enzymatic reaction mixture at both 37°C and pH 5.6.

Finally, Amino acid composition of the pure

glucose oxidase was also characterized by being rich in serine, glycine, glutamic acid, aspartic acid and alanine.

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