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Original Research Article

Purification of Glycolate Oxidase Enzymes from Peasm (*Pisum sativum* l.) and Sorghum (*Sorghum sudanense* J.) Using Ion-Exchange Chromatography and their Physicochemical Characteristics

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ABSTRACT

The glycolate oxidase enzyme, a core photorespiratory enzyme in green plants, oxidizes glycolate to glyoxylate, playing an adaptive role in roots during hypoxia to normoxia transition. The present study aimed to purify glycolate oxidase enzymes from peas (*Pisum sativum*L.) and sorghum (*Sorghum sudanense* J.), and investigate their physicochemical and regulatory properties. The activity of one of the glycolate oxidase isoforms (LCO-like glycolate oxidase) was induced in pea and sorghum leaves. The enzymes were purified to an electrophoretically homogeneous condition. The purified enzymes' specific activity, molecular mass, and physicochemical characteristics were determined. The results showed that the LCO-like glycolate oxidase enzyme from pea leaves was 314.37E/mg proteins, the degree of purification was 207 times, and the yield was 11.5%. The sorghum leaf enzyme was 274.22E/mg proteins with the degree of purification and yield, estimated at 143 times and 13.8%, respectively. The molecular mass of the native enzymes was 182 and 168kDa for peas and sorghum, respectively. It was revealed that the enzyme consisted of four subunits with a molecular mass of 45 and 42kDa for glycolate oxidase from pea and sorghum leaves, respectively, indicating a homo-tetramer. The sorghum leaf enzyme exhibited a higher affinity for lactate substrate with an optimal temperature of 55°C and pH of 7.2. Meanwhile, the optimal temperature and pH of pea leaf enzyme were 50°C and 7.5, respectively. The availability of homogeneous specimens provides opportunities to investigate the role of LCO-like glycolate oxidase in the adaptive reactions of cellular plant metabolism.

Keywords: Gel chromatography, Glycolate oxidase, Ion-exchange chromatography, Electrophoresis, Kinetics.

Introduction

Glycolate oxidase enzyme (EC 1.1.3.15) is specifically common and well-known among mammals and plants. The enzyme oxidizes glycolate to glyoxylate, making it one of the core photo respiratory enzymes in green plants.[1-3] It was discovered that glycolate oxidase could also use lactate as a substrate, in addition to glycolate.[2]Furthermore, when comparing amino acid and nucleotide sequences, a great similarity was revealed between *Arabidopsis* and spinach glycolate oxidases and L-lactate-cytochrome c-yeast oxidoreductase enzymes (EC 1.1.2.3), which utilize lactate in anaerobic conditions.[4] Engqvist *et al*.,[5] reported that an enzyme identical to yeast LCOwas present in plants that represents a glycolate oxidase is oform (EC 1.1.3.15). They hypothesized that this isoform has a higher affinity for lactate than the standard glycolate oxidase substrate (glycolate). Glycolate oxidase plays an adaptive role in the roots during the plant transition from hypoxia to normoxia.[6, 7] The glycolate oxidase enzyme, which is important for plants, was discovered to be active in both pea and sorghum leaves.[8, 9]

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The current study focused on purifying glycolate oxidase enzymes from peas (Pisum sativum L.) and sorghum (Sorghum sudanense J.), as well as examining their physicochemical and regulatory properties.

The activity of one of the glycolate oxidase isoforms, specifically the LCO-like glycolate oxidase, was induced in the leaves of both pea and sorghum plants.

Materials and Methods

Sources and maintenance of plant materials

The plant materials, sorghum (*Sorghum sudanense* J.) and peas (*Pisum sativum* L.) were collected from College of Agricultural, University of Baghdad (from May 2023 to January 2024), also all the collected samples were identified at the same place. The plant materials were authenticated at the Department of Protective Plant; University of Baghdad, Iraq, voucher No. 259, dated December 12, 2023. Samples were classified, identified at College of Agriculture, University of Baghdad, (The plant was taken from the botanical garden of the College of Agriculture, University of Baghdad, where it is classified, identified, and approved by the Department of Agricultural Crops in the same college for the purpose of experiments and research conducted by universities and research centers in Iraq). Wood chip-filled plastic containers with germinated plants were incubated inside a LabTech (at College of Agriculture Labs) in plant growth chamber for 42 days under the following controlled conditions: 12/12 hours of light/darkness photoperiod at a temperature of 25°C. The plants were placed under dark conditions for 24 hours before the extraction to reduce the activity of other glycolate oxidase isoforms directly participating in photorespiration.

Purification of LCO-like glycolate oxidase from peas and sorghum leaves

The enzymes were purified by their extraction from homogenized plant leaves, followed by gel filtration on a Sephadex G-25 column (1.5×20 cm), ion-exchange chromatography with DEAE Sephacel (GE Healthcare, Sweden), and gel chromatography on a Sephadex G-200 (GE Healthcare, Sweden) column (2.0×40 cm) according to the methods described by [9] Dry Sephadex was presoaked in buffer solutions according to the procedure highlighted in Besselink and de Korte (2002).[10]

Measurement of LCO-like glycolate oxidase activity

To measure the activity of LCO-like glycolate oxidase, the reaction medium contained 2 $cm^3$of 50 MмTris-HCl-buffer (pH 7.5), 4 mM sodium lactate/glycolate, 0.5 mM EDTA, 0.01 mM FMN, 5 mM $MgCl_2$ and 4 mM phenylhydrazine. The activity of each fraction was measured with both substrates. The samples that displayed higher activity with lactate were collected for further analysis. The protein's molecular mass was measured using gel filtration on a column of Sephadex G-200, which was calibrated with Dextran blue (2000 kDa). The value was calculated using the formula (1):

$$IgMr = 6.698 - 0.987\,(V_e)V_0 \quad \ldots\ldots\ldots\ldots\ 1$$

Where Ve is the elution volume of protein and Vo is the void volume.

Determination of the molecular mass of LCO-like glycolate oxidase

The molecular mass of the subunits was determined using 12% PAAG electrophoresis in the presence of Ds-Na. The following set of standard proteins (kDa) was used as molecular mass markers: β-galactosidase (116.0), BSA (66.2), ovalbumin (45.0), LDH (35.0),REase Bspl98 (25.0),β-lactoglobulin (18.4),and lysozyme (14.4). The gel was stained with silver nitrate based on the procedure described by Jain (2010).[11] The electrophoresis of LCO-likeglycolate oxidase was also performed in 8% PAAG using the method described byVershinina (2023).[12] Silver nitrate was applied to stain the gel, and the specific enzyme was measured using the tetrazolium method.[10] Proteins were measured using the Lowry method.

Statistical analysis

The variation statistical method was applied to determine the reliability of the research data. The data were analyzed using standard statistical methods. Statistically reliable differences were considered at $p \leq 0.05$. The data that were processed using the linear and parabolic approximation software was used to plot the curves.

Results and Discussion

Separation of the enzymes duringtheir highest activity stage is importantfor achieving the research objective. Therefore, the leaves of mature peas and sorghum plants were used to obtain highly purified LCO-likeglycolate oxidase. The results obtained from the purification of LCO-like glycolate oxidase from peas and sorghum leaves are presented in Tables 1 and 2, respectively. Figures 1 and 2 show that PAAG had one protein component with R_f=0.63 for peas and another with R_f=0.6 for sorghum. The electrophoresis under denaturing conditions was also employed with standard proteins to determine the molecular mass of the enzyme subunits, which were 45 and 42kDa for peas and sorghum, respectively (Table 3). Figure 3 depicts the determination of LCO-like glycolate oxidase of lactate and glycolate from peas and sorghum leaves. In contrast, Figure 4 highlights the parameters influencing the reaction rate between the enzymes purified from sorghum and peas.

During the multiphase purification approach, homogenous specimens of the LCO-like glycolate oxidase enzymes from pea and sorghum leaves were purified by electrophoresis. The specific activity was 274.22and 314.37E/mg, and the yield was 13.81 and 11.54% for sorghum and peas, respectively. Several methods provide effective purification of various substances.[13-15]

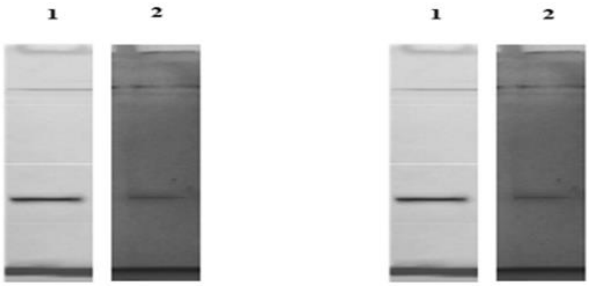

Figure 1: Electropherogram of LCO-like glycolate oxidase. A: *Pisum sativum*L.;b:*Sorghumsudanense* J.; 1: Specific measurement using the tetrazolium method; 2:Protein measurement using the universal stainer.

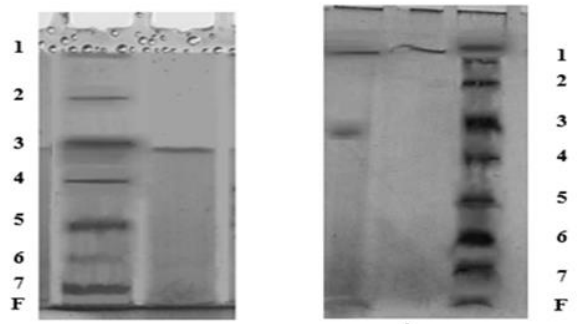

Figure 2: Ds-Na-electropherogram of LCO-like glycolate oxidase in 12% polyacrylamide gel based on the Laemmli method.
a:*Sorghum sudanense* J.;b:*Pisumsativum* L

However, the application of ion-exchange chromatography on DEAE Sephacelcontributed to obtaining a homogeneous specimen of LCO-like glycolate oxidase. The highest glycolate oxidase activity with lactate was desorbed from the carrier when the elution was 150-200mM NaCl. Protein components (R_f=0.63 for peas and R_f=0.6 for sorghum) were observed in PAAG using universal staining with silver nitrate. The tetrazolium method for measuring the specific activity of *LCO*-like glycolate oxidase showed that purified proteins demonstrated active glycolate oxidase. The findings indicated that the purified specimens were electrophoretically homogeneous, while the numbers correspond to the molecular mass of markers, such as *β*-galactosidase (116.0kDa), BSA (66.2kDa), ovalbumin (45.0kDa), LDH (35.0kDa), REase Bspl98 (25.0kDa), *β-lactoglobulin*, and lysozyme (14.4kDa).[16,17] The 238μM for peas and 143μM for sorghum (Figure 3), indicated that this enzyme had a high affinity for lactate. The findings are consistent with previously reported analyses of LCO-like glycolate oxidase from rice plants for which the Km value of lactate was 470μM, and *Arabidopsis*, for which this value was 420 μM[17, 18].Gel filtration on a Sephadex G-200 column was used to determine the molecular mass of the enzymes, which was 185 for peas and 168kDa for sorghum (Figure 3). The Michaelis constant of glycolate was 833μM for sorghum leaves and 588μM for peas. The Km values for glycolate in rice plant and *Arabidopsis* were 613 and 144μM, respectively.Electrophoresis under denaturing conditions was also performed with certain standard proteins to estimate the molecular mass of the analyzed enzyme subunits, which equaled 45 and 42kDa for peas and sorghum, respectively(Table 3). The results suggested that glycolate oxidase is a conservative enzyme, representing a tetramer with a molecular subunitwithamass ranging from 40 to 45kDa.The optimalpHvalue of the medium was determined within the TrisHCl buffer range of 5.0-9.5 and equaled 7.2 and 7.5 for achieving the activity of the LCO-like glycolate oxidase enzyme from peas and sorghum, respectively (Figure 4). The optimal temperature was determined within the range of 20-75°C and equaled 55 and 55°C for peas and sorghum enzymes, respectively. According to Zhang *et al*. (2017),[19] the optimal pH value for catalyzing the reaction of LCO-like rice glycolate oxidase was 7.8, and the enzyme's optimal temperature was 42°C.

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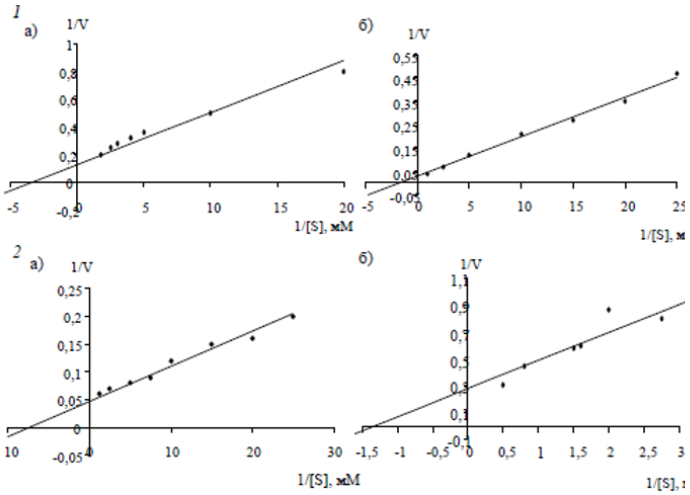

Figure 3: Km values for LCO-like glycolate oxidase a: Lactate; b:Glycolate; 1: *Pisum sativum* L. leaves; 2: *Sorghum sudanense* J. leaves.

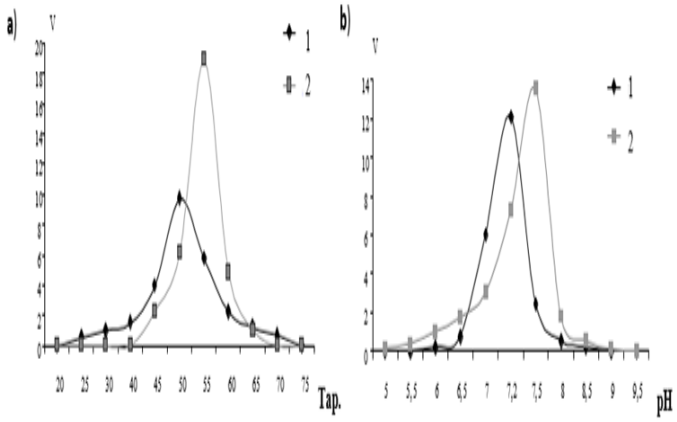

Figure 4: Reaction rate ofLCO-like glycolate oxidaseunder lactate oxidation conditions.
a: temperature; b: pH;1:*Pisum sativum* L.; 2:*Sorghum sudanense* J.

Table 1: Purification of LCO-like glycolate oxidasefrompea leaves.

Purification stage	Total volume (cm^3)	Total activity (E)	Proteins (mg)	Specific activity (E/mg)	Purification degree	Yield, (%)
Supernatant	18.0	137.32±4.12	90.72± 2.72	1.51±0.05	1.0	100
Ammonium sulphate precipitation (30-65%)	3.4	111.73±3.35	10.47±0.31	10.67±0.32	7.05	81.37
Gel filtration through Sephadex G-25	1.7	57.86±1.80	3.29±0.03	17.57±1.67	11.63	42.23
Ion-exchange chromatography on DEAE Sephacel	1.2	32.39±0.97	0.62±0.01	51.81±3.78	34.32	23.59
Gel chromatography on Sephadex G-200	0.9	15.84±0.48	0.11±0.02	143.59±4.43	95.09	11.53

n=3; p≤0.05

Table 2: Purification of LCO-like glycolate oxidasefromsorghumleaves

Purification stage	Totalvolume(c^3)	Total activity (E)	Proteins (mg)	Specific activity (E/mg)	Purification degree	Yield (%)
Supernatant	25.0	278.74±8.36	146.25±4.38	1.91±0.06	1.0	100
Ammonium sulphate precipitation (35%-65%)	4.0	204.17±6.91	20.02±0.60	10.20±0.31	5.35	73.24
Gel filtration through Sephadex G-25	2.3	175.00±4.77	5.72±0.17	30.59±0.92	16.05	62.78
Ion-exchange chromatography on DEAE Sephacel	1.5	135.00±1.34	2.54±0.02	53.24±4.36	27.87	48.57
Gel chromatography on Sephadex G-200	1.0	38.50±1.15	0.14±0.01	274.22±8.23	143.88	13.81

n=3; p≤0.05

Table 3: Molecular mass and subunit structure of LCO-like glycolate oxidase purified from *Pisum sativum* L. and *Sorghum sudanense* J.

Plant sample	Molecular mass (kDa)	Number of subunits	Molecular subunit mass (kDa)
Pisum sativum L.	182.0±2.7	4	45.0±1.9
Sorghum sudanense J.	168.0±2.5	4	42.0±1.3

Conclusion

Electrophoretically homogenous LCO-like glycolate oxidaseenzymes were purified from the peas and sorghum leaves using multiphase purification. The present study found that the enzymes represented a homo-tetramer. The analyzed sorghum leaf enzyme had a higher affinity for lactate substrate compared to the pea's enzyme, which could contribute to higher plant resistance. It was also expected that the optimal temperature for achieving the activity of the enzyme was 50°C for peas and 55°C for sorghum. Furthermore, the optimal pH for peas and sorghum was 7.5 and 7.2, respectively. The availability of homogeneous specimens opens upprospectsfor studying the involvement of LCO-like glycolate oxidase in adaptive reactions of cellular plant metabolism.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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