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EXPERIMENTAL ARTICLES

Purification of Pullulanase from *Aureobasidium pullulans*[1]

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Abstract—Purification and characterization of pullulanase from *Aureobasidium pullulans*. Pullulanase was purified by using gel—filtration column then on ion exchange using Q-sepharose column yielding a single peak. Purification was further carried out on SP-sepharose column. Molecular weight of pullulanase from *A. pullulans* was found to be about 73 KDa on the SDS-PAGE 10%. Native-PAGE 10% showed the activity of pullulanase, using polyacrylamide gel containing pullulan. Hydrolysis products from pullulanase activity with soluble starch, glycogen and pullulan on thin layer chromatography appeared as one band which is maltotriose, while α-amylase with soluble starch and glycogen showed two bands which are maltose and maltotriose but α-amylase gave negative result with pullulan on TLC chromatography only. Pullulanase could degrade α-1,6 glycosidic linkage of the previous substrates, while amylase could degrade α-1,4 glycosidic linkage of glycogen, soluble starch and pullulan. MALDI-Ms was employed to deduce protein sequence of pullulanase.

Keywords: *Aureobasidium pullulans*, Pullulanase, SDS-PAGE, Native-PAGE, Thin layer chromatography, Q-sepharose, SP-sepharose, MALDI-Ms.

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Pullulanase (EC 3.2.1.41) catalyzes the hydrolysis of 1-6-α-D-glucosidic linkages in pullulan (a linear polymer of α-1-6-linked maltotriose units occasionally some of maltotriose units are replaced by maltotetraose units). The α-1,6 bonds in amylopectin and pullulan are hydrolyzed by pullulanases which are enzymes belonging to glycosyl hydrolase family 57 [1]. α-Amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyze the α-1,4-linkages. Pullulanases do not show activity against linear (α-1,4-linked) oligosaccharides, and α-amylases show no activity against pullulan [2]. A cell-associated pullulanase from *Thermus aquaticus* YT-1 has been detergent-solubilized and purified by gel filtration and ion-exchange chromatography. Molecular weight was determined as 83 000 KDa by SDS-PAGE and 80000 KDa by steric exclusion chromatography [3]. After electrophoresis, active pullulanase bands from *Klebsiella aerogenes* in acrylamide gels were detected by overlaying and then incubating the gel on replica gel containing 2.5% pullulan—reactive red conjugate and 2.1% agar. The enzyme activity was revealed as a clear band against a red background on replica gel [4]. Using the susceptibility of pullulan CH-1 to hydrolysis catalysed by porcine α-amylase (EC 3.2.1.1) the polysaccharide was cleaved and the fragments obtained were fractionated by gel-permeation chromatography. The heterogeneous size of the fragments indicates that there is no apparent regular distribution of tetrasaccharide units in the pullulan chain. Enzymatic digestion of pullulan CH-1 using pullulanase (EC 3.2.1.41) from *A. pullulans* catalyses the hydrolysis of 1,6 α-D-glycosidic linkages, followed by gel-permeation chromatography of the resulting digest confirmed these results as did preparative paper chromatography and proved the presence of two components in the hydrolysate. The most abundant were shown to be maltotriose and maltotetraose [5].

MATERIALS AND METHODS

Growth Conditions of Aureobasidium pullulans

Czapek's medium ($NaNO_3$ 2g/l, KH_2PO_4 1g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4$ 0.001g/l), plus yeast extract 1g/l and sucrose 100 g/l at pH 5.5 was incubated for 5 days at 25°C was used as growth medium. 500 ml of the medium were dispensed into each of 1000 ml flasks, add five discs size (0.5 cm) in each flask and Shaken culture is used.

Enzyme Activity Assay

Pullulanase activity was determined using starch (0.6 mg/ml) as a substrate and iodine as an indicator (0.1% I_2. 0.5% KI). The control sample was 1 ml 50 mM acetate buffer pH 5.0 + 0.5 ml starch, but the

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crude sample was 1 ml enzyme (3.5 mg/ml) containing solution in 50 mM acetate buffer pH 5.0 + 0.5 ml starch and both were incubated overnight at 37°C. The colour absorbance was measured using spectrophotometer (Jenway 6305) at 660 nm using iodine test [6] by comparing the diffrence between zero time (A_0) and time-incubated sample (A_t).

Extraction and Purification of Pullulanase

Extraction of pullulanase. Precipitation of protein from mycelia and spores was made by adding acetone double volume. Sample was kept overnight, centrifuged at 10.000 rpm for 10 min at 4°C and acetone removed by air-drying then the protein was redissolved with 20 mM acetate buffer. Samples were dialyzed in a dialysis bag immersed in 50 mM acetate buffer, pH 5.0, overnight with gentle stirring at 4°C. The sample of protein was concentrated using polyethylene glycol (6000 MWt.)

Pullulanase purification using gel—filtration (sephadex G-100). Gel matrix (sephadex G-100) in sodium acetate 50 mM, pH 5.0 was used for packing the column, dissolving and applying the protein sample, fractions were collected (1 ml), the concentration was measured using spectrophotometer at A_{280} nm. Positive fractions were concentrated using dialyzing bag in PEG (6 000 MWt) and enzyme activity was assayed.

Pullulanase purification using ion—exchange chromatography: Q-sepharose: (GE, Hitrap Q strong anion exchanger): 17-5156-01 The protein will bind to an anion exchange resin if the pH buffer is higher than its isoelectric point (PI). Tris HC1 buffer 20 mM, pH 7.0 was used as mobile phase with increasing gradient of 100 mM–1M KCl applied to 5 ml Q-sepharose column (GE Company) using parastaltic pump (HBI, multistaltic pump), flow rate 1 ml/min., then fractions (1 ml each) were collected, measured using spectrophotometer at A_{280} nm. The positive fractions were collected and concentrated by PEG (6000 MWt), and enzyme activity was assayed.

***(SP-sepharose)*:** (GE, Hitrap SP strong cation exchanger): 17-5157-01 The protein will bind to cation exchange resin at lower pH buffer than its isoelectric point (PI). Sodium acetate buffer 50 mM, pH 4.0 was used as mobile liquid phase with a gradient of 200 mM–1 M NaCl applied to 5 ml SP-sepharose column (GE Company) using parastaltic pump (HBI, multistaltic pump) flow rate 1 ml/min, then fractions were measured using spectrophotometer at A_{280} nm. The positive fractions were collected and concentrated by PEG (6000 MWt).

Calculation of total protein. Calculation of the amount of protein from Crude protein, sephadex G-100, Q-sepharose and SP-sepharose employing biodiagnostic Kit. and colorimetric method was performed using spectrophotometer at A_{550} nm (Jenway 6305).

Electrophoretic Analyses

Determination of molecular weight by SDS-PAGE: 10% SDS-PAGE was performed using the method of protein bands [7], which were revealed by staining with G-250 silver blue staining [8].

A specific detection of pullulanase on native-PAGE. Electrophoresis was carried out at 4°C with a constant current of 20 mA using 10% polyacrylamide gel which contained 0.4 mg/ml of pullulan as specific substrate. After electrophoresis, the gel was soaked in sodium acetate buffer (pH 5.0), incubated for two days at 37°C, rinsed with distilled water, and pullulanase activity was determined by immersing the gel in Congo Red 1g/l for 30 min, then in iodine solution (1% I_2, 10% KI in 50% methanol) for 15 min.

Thin Layer Chromatography

Analyses of hydrolysis products of pullulanase and amylase on thin chromatography. Purified enzyme was used to compare the enzyme activity of pullulanase and amylase on pullulan, glycogen and soluble starch. 30 µl of purified enzymes were added to 50 µl of 0.4 mg/ml substrate in 50 mM sodium acetate buffer, (pH 5.0), incubated at 37°C for 24 h, mixture was separated on TLC plate (Merck, Silica gel 60) and detected by spraying with 5% sulphuric acid in methanol [9].

Application and activity of pullulanase on different substrates. Starch, pullulan and glycogen agar medium (0.4 mg/ml), then a well was made of about 0.5 cm, then 100 µl of pullulanase or amylase (human saliva) were added to the well. Incubated at 37°C others at 50°C for 48 h, staining with 1 g/l Congo Red for 20 min and then iodine for 10 min.

MALDI-Ms

MALDI-Ms was used to deduce the protein sequence of pullulanase.

RESULTS

Crude protein recorded concentrated 35 mg/ml, Purification of pullulanase was successfully carried out by sephadex G-100 using 50 mM sodium acetate buffer pH 5.0 showed concentrated 15 mg/ml. Two peaks for pullulanase activity were revealed (Fig. 1).

Further purification was performed using ion exchange chromatography: Firstly using Q-sepharose column (strong anion exchanger) and pullulanase was eluted at, 600 mM KCl in 20 mM Tris HCl, pH 7.0 which is higher than the isoelectric point of the protein shown concentrated 0.6 mg/ml (Fig. 2), Secondly

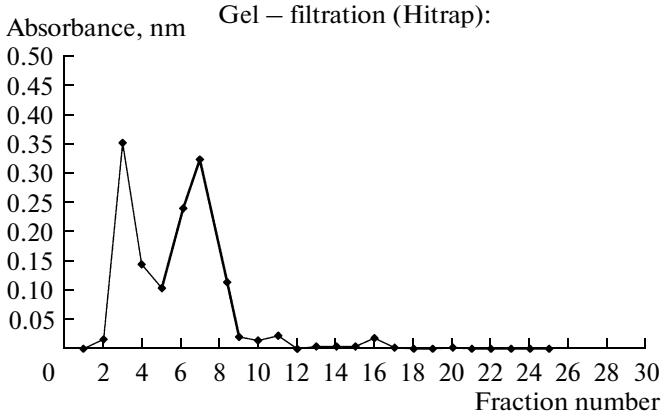


Fig. 1. Fraction number of protein samples from gel-filtration (sephadex G-100) 50 Mm sodium acetate buffer, pH 5.0.

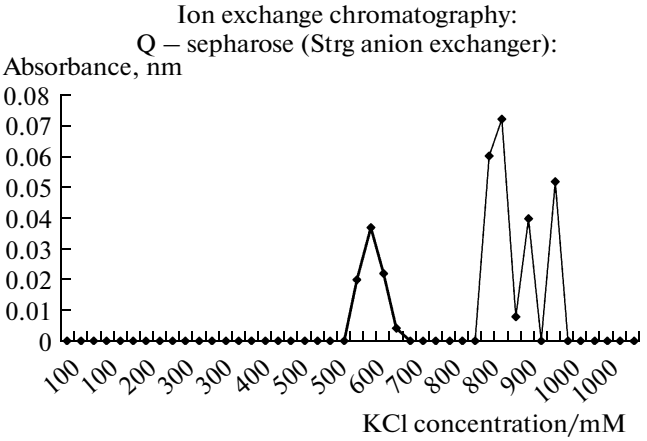


Fig. 2. Fractions of protein samples from Q-sepharose column with 20 mM Tris HCl, pH 7.0 plus KCl from 100 mM : 1000 mM.

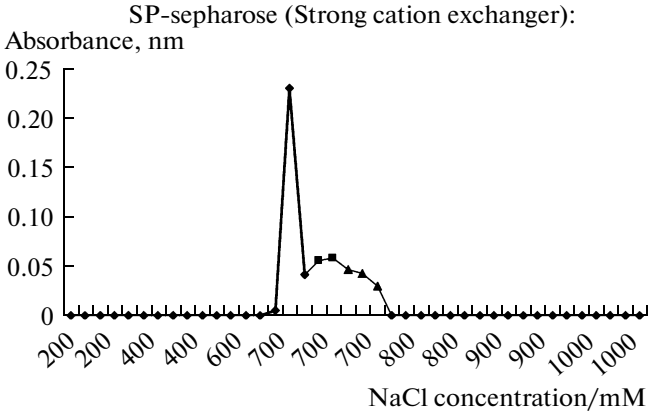


Fig. 3. Fractions of protein samples from SP-sepharose column with 50 mM sodium acetate buffer, pH 4.0, NaCl from 200 mM : 1000 mM.

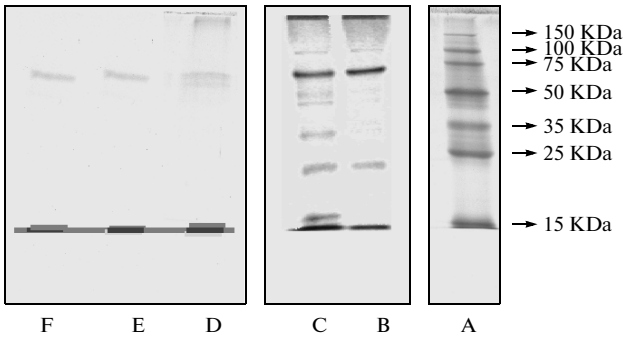


Fig. 4. 10% SDS-PAGE for purification steps of pullulanase from *Aureobasidium pullulans*
A: Control: standart protein markers.
B: Crude enzyme of Aswan isolate.
C: Crude enzyme of Fayoum isolate.
D: Peak fraction from sephadex G-100.

pullulanase was further purified with SP-sepharose (strong cation exchanger) and was eluted at 700 mM NaCl with 50 mM sodium acetate, pH 4.0, which is lower than the isoelectric point, showed concentrated 0.3 mg/ml (Fig. 3).

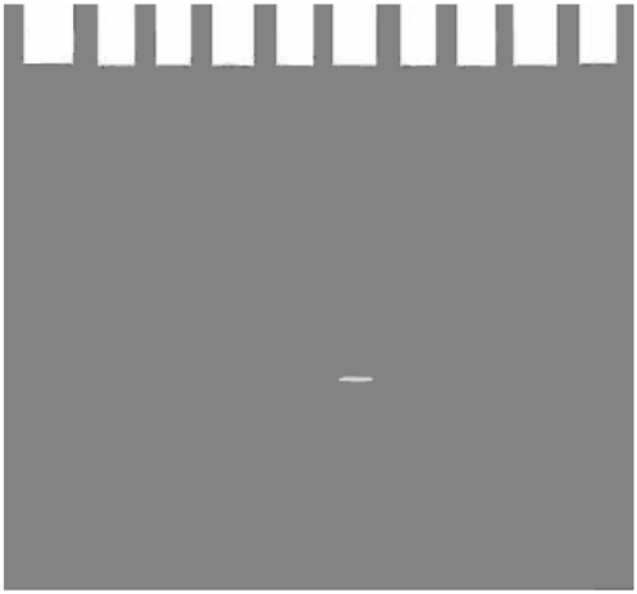
Pullulanase was indicated as a single protein band at molecular weight of about 73 KDa on the 10% SDS-PAGE (Fig. 4).

Activity of pullulanase on native—PAGE gel 10% containing pullulan which was stained with Congo Red and followed by iodine solution, appeared as a clear band on dark red background (Fig. 5).

TLC analysis of pullulanase revealed maltotriose (R_f 0.57 and 0.49) (Fig. 6) as the end product of soluble starch, glycogen, and pullulan Molecular weight of pullulanase was determined to be about 73 KDa on 10% SDS-PAGE gel (Fig. 4).

TLC analyses of α-amylase revealed maltose and maltotriose (R_f 0.57 and 0.49) as the end products of soluble starch and glycogen, but no hydrolysis products were detected with pullulan (Fig. 7 and table).

In our experiment, the activity of pullulanase and amylase was compared at 37°C on different substrates using colour indicators as follows: soluble starch with pullulanase was stained light blue with iodine; soluble starch with amylase gave no colour with iodine; pullulan and glycogen with pullulanase was stained light red with Cong Red plus iodine with dark background. This means that pullulanase can hydrolyze pullulan and glycogen. Pullulan and glycogen with amylase was stained light red with Congo Red plus iodine (small cycle); this means that amylase can hydrolyze pullulan and glycogen. At 50°C, pullulanase showed the same results with less activity than at 37°C (Fig. 8).



Qulitative analysis of pullulanase using Native-PAGE

Fig. 5. Activity of pullulanase on 10% polyacrylamide gel containing pullulan and staining with Congo Red, then Iodine solution.

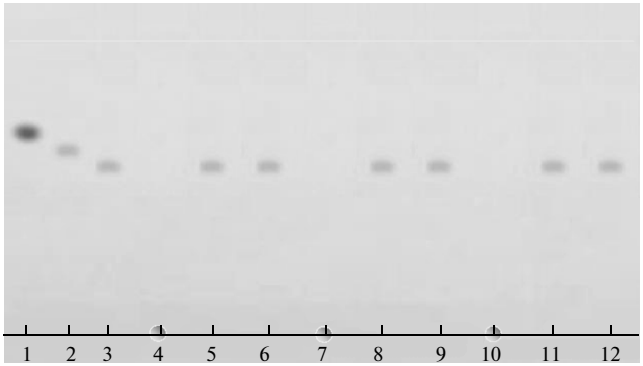


Fig. 6. Analyses of hydrolysis products of Pullulanase from Fayoum and Aswan isolates by thin layer chromatography.
1. Glucose (Standard); 2. Maltose (Standard); 3. Maltotriose (Standard); 4. Control of pullulan; 5. Pullulan + Pullulanase (from Aswan isolate); 6. Pullulan + Pullulanase (from Fayoum isolate); 7. Control of soluble starch; 8. Soluble starch + Pullulanase (from Aswan isolate); 9. Soluble starch + Pullulanase (from Fayoum isolate); 10. Control of glycogen; 11. Glycogen + Pullulanase (from Aswan isolate); 12. Glycogen + Pullulanase (from Fayoum isolate).

MALDI-Ms was used to deduce protein sequence of pullulanase. (Fig. 9). Protein score that observed match is greater than 67, Shown similarity scored 94 with RBS_HORVU Ribulose bisphosphate carboxylase small chain, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunit)—*Hordeum vulgare* (Barley) and matched peptides with Bold Red.

DISCUSSION

Purification of pullulanase was successfully carried out by sephadex G-100 using 50 mM sodium acetate buffer with pH 5.0, that revealed two peaks for pullulanase activity. Further purification was performed using ion exchange chromatography: Firstly using Q-sepharose column (strong anion exchanger) and pullulanase was eluted at 600 mM KCl in 20 mM Tris HCl, pH 7.0 which was higher than the isoelectric point of the protein, and secondly pullulanase was further purified with SP-sepharose (strong cation exchanger) and was eluted at 700 mM NaCl with 50 mM sodium acetate with pH 4.0 which was lower than the isoelectric point, that showed maximum activity.

Molecular weight of pullulanase was determined to be about 73 KDa on 10% SDS-PAGE gel. Molecular

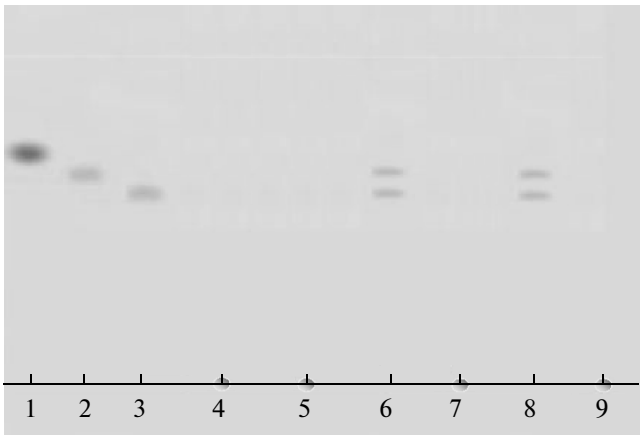


Fig. 7. Analyses of hydrolysis products of amylase by thin layer chromatography.
1. Glucose (Standard); 2. Maltose (Standard); 3. Maltotriose (Standard); 4. Pullulan + amylase; 5. Control of pullulan; 6. Soluble starch + amylase; 7. Control of soluble starch; 8. Glycogen + amylase; 9. Control of glycogen.

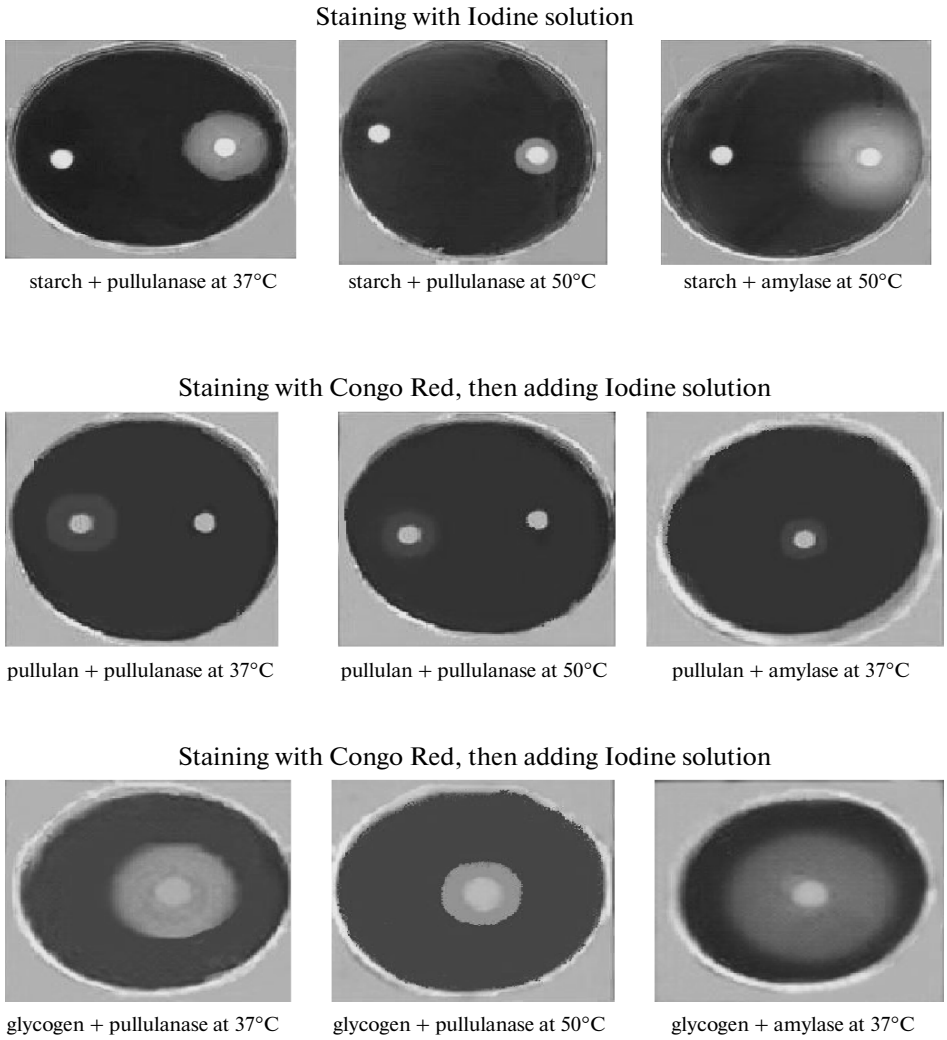


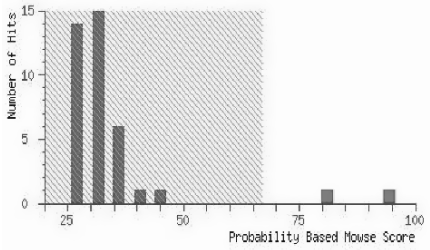
Fig. 8. The activity of pullulanase at 37°C and 50°C and amylase at 37°C on starch, pullulan and glycogen after two days.

Mascot Search Results

User : ali
Email : jakarin.ali@ncl.ac.uk
Search title : s1
Database : SwissProt 52.3 (264492 sequences; 96880444 residues)
Timestamp : 27 Apr 2007 at 10:43:44 GMT
Top Score : 94 for RBS_HORVU, Ribulose bisphosphate carboxylase small chain, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunit) - Hord

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 67 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help
Significance threshold p< 0.05 Max. number of hits 20

Re-Search All | Search Unmatched

1. RBS_HORVU **Mass:** 19408 **Score:** 94 **Expect:** 9.6e-05 **Queries matched:** 12
Ribulose bisphosphate carboxylase small chain, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunit) - Hordeum vulgare (Barley)
RBS3_WHEAT **Mass:** 13046 **Score:** 75 **Expect:** 0.009 **Queries matched:** 9
Ribulose bisphosphate carboxylase small chain clone 512 (EC 4.1.1.39) (RuBisCO small subunit) (Fragment) - Triticum aestivum (Wheat)
RBS5_LEMGI **Mass:** 19790 **Score:** 38 **Expect:** 44 **Queries matched:** 5

Protein View

Match to: **RBS_HORVU** Score: **94** Expect: **9.6e-05**
Ribulose bisphosphate carboxylase small chain, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunit) - Hordeum vulgare (Barley)

Nominal mass (M_r): **19408**; Calculated pI value: **8.98**
NCBI BLAST search of RBS_HORVU against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Hordeum vulgare

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **62**
Number of mass values matched: **12**
Sequence Coverage: **58%**

Matched peptides shown in **Bold Red**

```
     1 MAPTVMASSA TSVAPFQGLK STAGLPVSRR SNASSASVSN GGRIRCMQVM
    51 PIEGIKKFET LSYLPPLSTE ALLKQVDYLI RSKWVPCLEF SKVGFIFREH
   101 NASPGYYDGR YWTMWKLPMF GCTDATQVLN EVEEVKKEYP DAYVRIIGFD
   151 NMRQVQCVSF IAFKPPGCQE SGKA
```

Show predicted peptides also

Sort Peptides By (•) Residue Number ( ) Increasing Mass ( ) Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
1 - 20	1994.0109	1993.0036	1993.0013	1	0	-.MAPTVMASSATSVAPFQGLK.S
57 - 74	2050.1967	2049.1895	2049.1398	24	1	K.KFETLSYLPPLSTEALLK.Q
58 - 74	1922.0906	1921.0833	1921.0448	20	0	K.FETLSYLPPLSTEALLK.Q
84 - 92	1165.6157	1164.6084	1164.5638	38	0	K.WVPCLEFSK.V Carbamidomethyl (C)
99 - 110	1365.6239	1364.6166	1364.5745	31	0	R.EHNASPGYYDGR.Y
111 - 116	914.4490	913.4418	913.4156	29	0	R.YWTMWK.L
111 - 116	930.4485	929.4412	929.4106	33	0	R.YWTMWK.L Oxidation (M)
117 - 137	2367.1720	2366.1648	2366.1498	6	1	K.LPMFGCTDATQVLNEVEEVKK.E Oxidation (M)
117 - 137	2408.2383	2407.2310	2407.1763	23	1	K.LPMFGCTDATQVLNEVEEVKK.E Carbamidomethyl (C)
117 - 137	2424.2246	2423.2173	2423.1712	19	1	K.LPMFGCTDATQVLNEVEEVKK.E Carbamidomethyl (C); Oxidation (M)
138 - 145	1012.4969	1011.4897	1011.4661	23	0	K.EYPDAYVR.I
146 - 153	965.5262	964.5190	964.4800	40	0	R.IIGFDNMR.Q

No match to: 706.5565, 801.5320, 817.4862, 833.0902, 833.4514, 842.5369, 845.5354, 855.0580, 861.5159, 865.0455, 871.0505, 877.4799, 886.432

Fig. 9. MALDI—Ms results.

Analyses of hydrolysis products by thin layer chromatography

Samples	R_f value
Glucose (Standard)	0.64
Maltose (Standard)	0.57
Maltotriose (Standard)	0.49
Soluble starch + pullulanase (Aswan isolate)	0.49
Soluble starch + pullulanase (Fayoum isolate)	0.49
Pullulan + pullulanase (Aswan isolate)	0.49
Pullulan + pullulanase (Fayoum isolate)	0.49
Glycogen + pullulanase (Aswan isolate)	0.49
Glycogen + pullulanase (from Fayoum)	0.49
Pullulan + amylase	No result
Soluble starch + amylase (I)	0.57
Soluble starch + amylase (II)	0.49
Glycogen + amylase (I)	0.57
Glycogen + amylase (II)	0.49

weight of pullulanase from *Thermus aquaticus* YT-1 which was purified by gel filtration and ion exchange chromatography, was found to be 83 KDa by SDS–PAGE [3].

Activity of pullulanase on native-PAGE gel 10% containing pullulan which was stained with Congo Red and followed by iodine solution, appeared as a clear band on dark red background. Activity of pullulanase from *Klebsiella aerogenes* appeared also as a clear band against a red background on replica of native gel containing 2.5% pullulan-reactive red conjugated and 2.1% agar [4].

TLC analysis of pullulanase revealed maltotriose (R_f 0.49) as the end product of soluble starch, glycogen, and pullulan. The α-1,6 glycosidic bonds in amylopectin and pullulan are hydrolyzed by pullulanase which are enzymes belonging to glycosyl hydrolase family 57 [1]. Digestion of the crude expolysaccharide with commercial pullulanase gave maltotriose as the major product by TLC analysis [10]. Enzymatic digestion of pullulan CH-1 using pullulanase from *Aureobasidium pullulans* proved the presence of maltotriose in the hydrolysate of pullulans. [5]. Pullulanase from alkaliphilic *Bacillus* species S-1, produced maltotriose only as a hydrolysate product of pullulan, whereas; amylopectin yielded limited α-β-dextrin and glycogen and small amounts of glucose and maltose were detected [11]. Also, pullulanase from *Bacillus circulans* SV-98 was found to produce maltotriose from pullulan [12].

In the present investigation TLC analysis of α-amylase activity revealed maltose and maltotriose (R_f 0.57 and 0.49) as the end products of soluble starch and glycogen, but no hydrolysis products were detected with pullulan. Paper chromatography indicated that hydrolysis of pullulan CH-1 by α-amylase (EC 3.2.1.1) yielded at least four heterogeneous fragments that must have relatively high molecular weights since they did not move far from the starting line with the solvent used [5].

In present experiment, the activity of pullulanase and amylase was compared at 37°C on different substrates using colour indicators as follows: soluble starch with pullulanase was stained light blue with iodine; soluble starch with amylase gave no colour with iodine. Pullulan and glycogen with pullulanase was stained light red with Cong Red + iodine with dark background; this means that pullulanase can hydrolyze pullulan and glycogen. Pullulan and glycogen with stained light red with Congo Red + iodine (small cycle); this means that amylase can hydrolyze pullulan and glycogen. At 50°C, pullulanase showed the same results with less activity than at 37°C. Pullulanase type I from alkaliphilic *Bacillus* sp. S-1 hydrolyzes the α-1,6-glycosidic bonds in soluble starch, increased the amounts of α-1,4-linked amylose which yields more intensely blue colored conjugate with iodine. Thus, in contrast, amylolytic enzymes give white bands on the lightly stained background because they remove amylose [13]. Amylase could degrade the α-1,4 glycosidic linkages of pullulan but not appeared on TLC as reported in [5]. This hydrolysis must have relatively high molecular masses since they did not move far from the starting line with the solvent used, but we managed to show the hydrolysis of pullulan by α-amylase on Petri dishes only.

MALDI-Ms was recorded to deduce protein sequence of pullulanase. Shown similarity scored 94 with RBS_HORVU Ribulose bisphosphate carboxylase small chain, chloroplast precursor (EC 4.1.1.39) (RuBisCO small subunit)—*Hordeum vulgare* (Barley).

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