FAT *HYDROLYSIS* BY *BACILLUS SUBTILIS* LIPASE AND SOME PROPERTIES OF THIS ENZYME

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Abstract

The best saturation for lipase precipitation by ammonium sulphate was found to be 0.8, which gave the highest yield, being 90.9% with specific activity and degree of purification of 70.1 μmol FFA/mg protein/min. and 8.75 folds, respectively. By using 0.8 saturation of ammonium sulphate, pH 6.0 was found as the best pH value for enzyme precipitation. The yield of lipase at such pH value was 76.36%. The effects of pH and temperature on lipase activity varied with the substrate. The optimum pH was in the range of pH 7.9; the optimum temperature was in the range 40-50°C in case of olive oil which was the best tested substrate. The pH stability curve at 4oC showed no discontinuities and had a maximum between pH 6.0 and 8.0 at 1, 24 and 48 hrs. Also at 21-50°C, the residual activity reached 88.1% and 47.6%, respectively. Moreover after 48 hrs, the residual activity showed 71.4 and 35.7%. respectively. The enzyme lost very little activity between 30-50°C and was also quite stable at 60°C, but it lost activity very rapidly at 80°C. The hydrolysis of oils by *Bacillus subtilis* lipase was also studied and the degree of hydrolysis at 40°C reached after 24 hrs 92.11, 84.62 and 79.79%, for olive, palm and coconut oils, respectively.

INTRODUCTION

Recently, preparation of microbial lipase have been produced industrially. It is therefore likely that in the foreseeable future, industrial processes will be developed for enzymatic hydrolysis of triglycerides. At present, number of lipases are available commercially; the enzmology of these lipases is well documented (Brockerhoff & Jensen, 1974 and Werdelmann & Schmid, 1982). There are however, few reports which deal with physicochemical properties of lipases, in comparison with reports available on other enzymes. This may be attributed to the unstability of purified lipases as general (Sugiura and Oikawa, 1977). However, to date there has been no published reports available on how *Bacillus subtilis* lipase could hydrolyze oils as compared with other lipases. Also, no published study show the

physicochemical properties of this enzyme.

The present study describes the partial purification, some properties and lipolysis of oilve, coconut and palm oil of *Bacillus subtilis* lipase.

MATERIALS AND METHODS

Organisms:

The microorganism used in this study was *Bacillus subtilis* (12). It was obtained from Microbiology Dept., Fac. of Agric., Mansoura Univ. Mansoura, Egypt.

Culture medium:

Sugiura *et al.* (1977) basal medium with some modification (Hauka *et al.*, 1997b) was used. It consisted of: meat extract 0.3%, polypeptone 1.5%, glucose 1.0%, KH_2PO_4 0.2%, KCl 0.05%, $MgSO_4$ 0.05%, olive oil 1.0%. The pH was adjusted to 6.0. After inoculation, the cultures were incubated at 30°C for 3 days with agitation (120 strokes/min) in a reciprocating shaker bath. The culture technique has been described by Hauka *et al.* (1997a). The cultures were centtrifuged at 8000 rpm for 15 min. and cell free extracts were used as lipase source.

Lipase assay:

Lipase activity was determined by the method of Oi *et al.* (1969) with some modification by Hauka *et al.* (1997a). The reaction mixture contained 5.0 ml of 5% olive oil emulsified in 9% acacia gum in distilled water, 0.1% sodium desoxycholate, 5.0 ml of 0.2 M Tris-H Cl buffer (pH 8.5), 2.0 ml of 0.2 M $CaCl_2$ solution, 1.0 ml enzyme solution, and 2.0 ml distilled water. After the incubation period under the assay condition (10 min), the total amount of liberated fatty acids was titrated against N/100 NaOH. The blank was the same assay mixture containing boiled enzyme.

Protein assay:

Protein concentration was measured by the method of Lowry *et al.* (1951) in comparison to boving serum albumin as standard.

Enzyme precipitation:

The enzyme culture filtrate was precipitated by using ammonium sulphate as

described by Tsujisaka *et al.* (1973) as follows: to the culture filtrate, solid ammonium sulphate was added up to 0.1 saturation. The mixture was left overnight at 4°C and the precipitate was centrifuged at 20000 rpm for 20 minutes in a cooling centrifuge. The experiment was repeated using ammonium sulphate at different saturations being 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0. The precipitate was dissolved in a minimum amount of distilled water and the activity of lipase was determined.

Also, the culture filtrate was precipitated using ammonium sulphate (0.8 saturation) at different pH values being 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The precipitation was carried out as follows: ten ml of culture filtrate were adjusted to different pHs and saturated with selected concentration of ammonium sulphate and left overnight at 4°C. The precipitate was collected by centrifugation at 20000 rpm in a cooling centrifuge at 4°C for 20 minutes, then dissolved in a little amount of distilled water (2 ml). The collected solution was used for determination of the enzyme activity.

pH stability:

To demonstrate stability at different pH levels, precipitated enzyme solution was brought to different pH values (pH 2-10) by addition of universal buffers and kept for 1, 24 and 48 hrs, at 4, 21 and 50°C. The activity of these pre-incubated samples was measured.

Temperature stability:

To investigate thermal stability, precipitated solution was kept at optimum pH for 10, 20, 30, 40, 50 and 60 minutes at different temperatures (30-100°C) and residual enzyme activity was measured.

Hydrolysis of oils:

The degree of hydrolysis of olive oil, coconut oil, and palm oil were determined according to the method described by Linfield *et al.* (1984). Acid value and saponification number were determined according to the A.O.A.C. (1970) of official method of analysis.

RESULTS AND DISCUSSION

Effect of ammonium sulphate on enzyme precipitation:

The results achieved on the effect of ammonium sulphate on enzyme precipitation are summarized in Table (1). Results indicate that yield of lipase increaed with increasing ammonium sulphate concentration up to 0.8 saturation. Also, the results reveal that the highest precipitation was obtained at the above degree of saturation. The specific activity obtained at this saturation level was the highest being 70.1 µmol FFA/mg protein/min. and the degree of purification was 8.75 folds. These results are in accordance with those obtained by Tsujisaka *et al.* (1973).

Table 1. Effect of ammonium sulphate concentration on the precipitation of *Bacillus subtilis* lipase (pH 7.35).

$(NH_4)_2SO_4$ saturation	Total activity in precipitated fraction (µmole FFA/100 ml/ min.)	Specific activity as µmole FFA/ mg protein/min	Degree of purification (fold)	Yield (%)
Crude enzyme	3300	8.01	1.00	--
0.1	660	16.02	2.00	20.0
0.2	860	20.87	2.61	26.1
0.3	980	22.90	2.86	29.7
0.4	1600	37.38	4.67	48.5
0.5	1800	42.06	5.25	54.5
0.6	2140	50.00	6.24	64.8
0.7	2600	60.75	7.58	78.8
0.8	3000	70.10	8.75	90.9
0.9	2720	63.55	7.93	82.4
1.0	2180	50.93	6.36	66.4

Effect of pH on the precipitation of lipase by ammonium sulphate:

Since proteins always show minimum solubility at their iso-electric point. It was necessary to investigate how much precipitation can be achieved by changing the pH value of the culture filtrate at 0.8 saturation. Maximum precipitation and yield of lipase was tested at different pH values as shown in Table (2). From the results, it is seen that the precipitation of maximum lipase was greatly affected by pH of culture filtrate used as enzyme source. By increasing the pH value of culture filtrate up to pH 6.0, the yield of precipitated enzyme was increased to 76.36%. The specific activity at pH 6.0 is 68.88 µmol FFA/mg protein/min. By increasing the pH value above 6.0, the yield of enzyme began to decrease. This means that ammonium sulphate at 0.8 saturation and pH 6.0 gives high amount of precipitated enzyme. Fukumoto *et al.* (1966) found that ammonium sulphate at pH 5.0 precipitated all lipases studied.

Table 2. Effect of pH on the precipitation of *Bacillus subtilis* lipase by ammonium sulphate (0.8 saturation) .

pH	Total activity in precipitated fraction (µmole FFA/1000 ml/min.)	Specific activity as µmole FFA/ mg protein/min.	Degree of purification (fold)	Yield (%)
Crude enzyme	3300	8.01	1.00	--
4	2250	52.57	6.56	68.18
5	2750	58.57	7.31	83.33
6	3150	68.88	8.60	96.36
7	3050	63.50	7.93	92.42
8	1850	47.06	5.88	56.06
9	1755	41.00	5.12	53.18

pH and temperature optima of *Bacillus subtilis* lipase during hydrolysis of different oils:

The influence of pH and temperature was studied by varying the reaction of buffer solution in this experiment. Universal buffers were used in order to cover the pH range 5.0 to 9.0 and reaction mixture in this experiment was incubated at 30, 40, 50 and 60°C for different oils, i.e. olive, corn, coconut, palm and butter oils. The data obtained are illustrated in Table (3).

The effect of pH and temperature on lipase activity varied with the type of substrate and depending on it. The pH optimum for lipase activity in olive oil and butter oil emulsions was pH 8.0 at 40°C as temperature optima. But, the temperature optima for enzyme activity in corn oil, coconut oil, and palm oil were 50°C at the optimum pH 8.0. Above and below of the optima pH and temperature, lipase activities were reduced greatly. For all oils at pH 5.0 at 30, 40, 50 and 60°C, there was little or no activity. These findings are similar to those obtained by Driessen & Stadhouders (1974) and Adams & Brawley (1981). These findings mean that heat resistant lipase would be quite active during non-refrigerated storage.

pH stability of *Bacillus subtilis* lipase:

As for pH stability of the enzyme, the results illustrated in Table (4) indicated that the enzyme is highly active at pH values between 6.0 and 8.0 at 4.0°C for 1, 24 and 48 hrs. In other words, the enzyme stability is highest in this pH range. Beyond this range of pH the enzyme activity decreased. Also, at 21 and 50°C, the residual activity reached to 88.1 and 47.6% after 1 hr., but after 24 hrs. the residual activity recorded 71.4 and 40.5%, respectively. Moreover, after 8 hrs. the residual ac-

Table 3. pH and temperature optima of *Bacillus subtilis* lipase during hydrolysis of different oils (enzyme activity expressed as µmole FFA/ mg protein/min.)

Temp °C / pH	Lipase activity																			
	Olive oil				Corn oil				Coconut oil				Palm oil				Butter fat			
	30	40	50	60	30	40	50	60	30	40	50	60	30	40	50	60	30	40	50	60
5	5	6	10	5	0	0	0	10	0	0	0	8	0	0	0	0	0	0	0	0
6	30	50	40	28	8	10	24	15	8	26	20	15	10	20	34	10	30	34	26	15
7	32	56	80	30	20	20	40	35	30	36	40	30	26	36	50	18	40	36	26	16
8	36	84	56	50	30	40	70	60	36	36	70	30	50	54	54	38	28	72	70	20
9	20	50	54	48	20	30	50	24	28	30	50	26	28	32	38	22	16	60	50	40

Table 4. pH stability* of *Bacillus subtilis* lipase.

Temperature	Residual lipase activity %								
	4°C			21°C			50°C		
Time / pH	1 hr.	24 hrs.	48 hrs.	1 hr.	24 hrs.	48 hrs.	1 hr.	24 hrs.	48 hrs.
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	60.0	38.1	21.4	47.6	35.7	16.7	23.8	21.4	0.0
6	83.3	71.4	71.4	83.3	59.5	59.5	40.5	35.7	28.6
7	90.5	76.2	71.4	85.7	66.7	64.3	42.9	38.1	31.0
8	95.2	83.3	71.4	88.1	71.4	71.4	47.6	40.5	35.7
9	59.2	35.7	35.7	42.9	35.7	33.3	23.8	21.4	11.9
10	26.2	16.7	11.9	21.4	11.9	9.5	21.4	11.9	9.5

* pH stability studies were performed by storing lipase at 4, 21 and 50°C for 1, 24 and 48 hrs. in buffer solutions of different pH values and measuring the residual lipase activity %.

tivity showed 71.4 and 35.7%, respectively. Data in line with our results and suppositions concerning enzyme stabilities were reported by Kosugi and Kamibayashi (1971).

Thermal stability:

Perhaps the most industrially significant feature of lipase is its remarkable heat stability. Therefore, thermal denaturation curves for *Bacillus Subtilis* lipase at optimum pH (8.5) are shown in Table (5). The enzyme lost very little activity between 30-50°C and was also quite stable at 60°C. The enzyme lost activity very rapidly at 80°C. The lack of enzyme activity at the higher degree of temperature might be explained on the basis of protein denaturation at such higher temperature. Our results are in close agreement with the findings of Fox & Stepaniak (1983) and Lamberet & Menassa (1983).

Table 5. Thermal stability* of *Bacillus subtilis* lipase.

Temperature / Time (min)	Residual lipase activity (%)					
	30°C	40°C	50°C	60°C	80°C	100°C
10	100.0	100.0	85.0	85.0	50.0	25.0
20	95.0	92.5	82.5	80.0	37.5	20.0
30	95.0	92.5	82.5	80.0	25.0	17.0
40	92.5	90.0	82.5	78.0	22.5	12.5
50	92.5	87.5	80.0	78.0	22.5	12.5
60	92.5	85.0	80.0	69.75	17.5	7.5

* Thermal stability studies were performed by placing the enzyme in optimum pH (tris-HCl buffer) for 10,20,30,40,50 and 60 min. at 30,40,50,60,80 and 100°C and measuring residual lipase activity (%).

Enzymatic fat hydrolysis:

Lipolysis of olive oil, and palm oil with *Bacillus subtilis* lipase was carried out at 40°C. Enzyme units used for fat hydrolysis are 3, 6,9, and 15 units at 1,2,4,8, 16 and 24 hrs. The degree of hydrolysis (DH%) are shown in Table (6). The results in Table (6) show that this enzyme can be able to hydrolyze these oils and the degree of hydrolysis of all oils increased with increasing the units of enzyme. DH% reached to 92.11, 84.62, and 79.79 for olive oi, palm oil, and coconut oil, respectively, after 24 hrs of hydrolysis by 15 units of enzyme. These findings are in accordance with those of Linfield *et al.* (1984).

Table 6. Hydrolysis of some oils with *Bacillus subtilis* lipase at different reaction times.

Reaction time hrs. / Enzyme unit	Degree of hydrolysis (%)																	
	Palm oil						Coconut oil						Olive oil					
	1	2	4	8	16	24	1	2	4	8	16	24	1	2	4	8	16	24
3	4.42	8.80	11.99	17.67	25.25	38.38	3.78	8.46	14.99	26.42	33.91	39.54	4.02	7.89	17.67	26.41	36.44	41.58
6	8.27	15.67	23.56	31.69	39.29	59.29	7.64	14.79	24.37	33.08	42.74	55.05	8.42	14.70	26.66	45.24	59.26	66.84
9	11.01	24.00	30.31	45.77	60.00	74.64	11.17	22.56	31.01	39.74	56.27	67.29	15.85	27.20	35.47	52.77	71.05	83.68
15	15.99	34.36	50.21	64.79	77.95	84.62	16.66	35.36	47.35	56.00	64.80	79.79	25.34	37.47	49.91	65.83	80.32	92.11

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تحليل الدهن بواسطة ليبيز الباسيليس سابتلس وبعض خصائص هذا الإنزيم

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حديثاً زاد الإهتمام بإستخدام الليبيز من المصادر المختلفة بصفة عامة ومن الميكروبات بصفة خاصة وذلك للأهمية المتزايدة يوماً بعد الآخر فى أيامنا الحالية للجوانب البيوتكنولوجية المتعددة لهذه الإنزيمات ولذلك تركز هذا البحث علي دراسة بعض خصائص وإستخدام ليباز الباسيليس سابتلس فى تحليل بعض الزيوت وقد خلصت الدراسة إلى :-

للحصول على أعلى ترسيب لليبيز من الراشح الخلوى وجد أن ٠,٨ تشبع من كبريتات الأمونيوم تعطى أعلى كمية ونشاط للإنزيم حيث وصلا إلى ٩٠,٩٠ ٪، ٧٠,١٠ ميكرومول أحماض دهنية حرة لكل مجم بروتين على الترتيب وقد بلغت درجة التنقية ٨,٧٥ مرة. وقد وجد أن زيادة تركيز أيون الأيدروجين للراشح الخلوى إلى ٦ تزيد كمية البروتين الإنزيمى المتحصل عليها.

تبين أيضا أن تركيز أيون الأيدروجين ٨ هو الأمثل لنشاط الإنزيم مع كل الزيوت المختبرة وأن درجة حرارة ٤٠ °م هى المثلى فى حالة زيت الزيتون والسمن البلدى ودرجة ٥٠ °م هى المثلى فى حالة زيت الذرة وزيت جوز الهند وزيت النخيل، وقد تميز الإنزيم بدرجة ثبات عالية عند تركيز أيون الأيدروجين بين ٦ - ٨ و تناقص النشاط خارج هذا المدى سواء عند درجات ٤ ، ٢١، ٥٠ °م لمدة ٢٤ و ٤٨ ساعة وقد توقف نشاط الإنزيم عند تركيز أيون أيدروجين ٥ مع درجات الحرارة والأوقات السابقة. تميز الإنزيم بثبات حرارى بين ٣٠ و ٥٠ °م حتى ٦٠ دقيقة إلا أن النشاط قد تضائل بإرتفاع درجة الحرارة عن ٦٠ °م وقد وجد أن معدل تحلل زيوت الزيتون والنخيل وجوز الهند وصل بعد ٢٤ ساعة إلى ٩٢,١١ و ٨٤,٦٢ و ٧٩,٧٩٪ على الترتيب بإستخدام خمسة عشر وحدة من الإنزيم.