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SOME PROPERTIES OF A LIPASE FROM *RHIZOPUS ARRHIZUS*

SEPARATION OF A GLYCOPEPTIDE BOUND TO THE ENZYME

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SUMMARY

The exocellular lipase from *Rhizopus arrhizus* is a glycoprotein of molecular weight around 43 000, containing 13–14 molecules of mannose and 2 molecules of hexosamine and a single N-terminal aspartic acid residue (or asparagine).

This lipase (lipase I) consists of 2 apparently noncovalently linked portions: a glycopeptide of molecular weight around 8500 and the enzyme protein. This latter can be easily freed from the glycoprotein by heating or by precipitation in the cold with 5% trichloroacetic acid.

The glycopeptide does not play any role in the catalytic function of the enzyme. Upon long storage of lipase I, this glycopeptide separates and lipase I is converted into a second active form, lipase II. This slow separation is paralleled by a fragmentation of the glycopeptide into peptides and shorter glycopeptides. The enzymic protein also seems to be partly degraded as shown by the disappearance of the single N-terminal aspartic acid (or asparagine) residue and the appearance of several other N-terminal residues in non-stoichiometric proportions.

INTRODUCTION

Recently there have been many studies on the lipases of microorganisms. Some of these lipases have been purified, and some information is available about their structure[1–3]. Other lipases have been prepared in order to compare their positional specificity[4] with the well-known specificity of pancreatic lipase[4,5].

In this laboratory studies on the lipase produced by the mold *Rhizopus arrhizus* have shown that this enzyme has the same positional specificity as pancreatic lipase[6] and that only the external chains of triglycerides are hydrolysed.

A purification technique for the lipase from *Rhizopus arrhizus* was described in a preceding publication[7]. This microorganism produces a single exocellular lipase (lipase I) which, in solution, is slowly transformed into another form nearly as active as the first one but slightly more cationic (lipase II). During filtration through Sephadex

G-100, these two lipases behave as proteins of molecular weights 40 000 ± 2000 and 30 000 ± 1500, respectively[7].

In the present study some of the structural properties of lipase I were determined after its stabilisation with 2 mM DFP. This form appears to result from a noncovalent association of an enzyme protein with a glycopeptide of molecular weight around 8000. The glycopeptide can be separated from the lipase by simple heating or by precipitation of the enzyme protein with 5% trichloroacetic acid in the cold. During the slow transformation of lipase I into lipase II, the glycopeptide part is also removed. However, the transformation is, in this case, probably enzymatic. It is paralleled by a fragmentation of the glycopeptide and by a limited degradation of the lipase which seems furthermore to affect its activity very little.

MATERIALS AND METHODS

Measurement of enzymatic activity

Lipase activity was measured at 37° and pH 8.0 by the automatic pH-stat titration of liberated fatty acid during the hydrolysis of long-chain triglycerides. The concentrated triglyceride emulsion is prepared by emulsifying 50 g of olive oil in 400 ml of a 2% solution of Methocel (60 HG, 50 Cps, Dow Chemical Co.) for 4 min in a Waring Blendor. To assay the enzyme, 6 ml of this emulsion were diluted to 20 ml with an aqueous solution of NaCl, $CaCl_2$ and bovine serum albumin (Cohn Fraction V), the final concentrations being 0.1 M, 5 mM and 0.05%, respectively. The enzyme is added in a volume of 20–300 μl. One lipase unit is the amount of enzyme which liberates 1 μmole of fatty acid per min under the conditions of the assay. The specific activity is the number of lipase units per mg of protein. Determinations of proteins were done by the colorimetric technique of LOWRY *et al.*[8], with bovine serum albumin (Fraction V) as the standard.

Preparation of lipases I and II

Lipase I was prepared from a lyophilised extract of the culture medium of *Rhizopus arrhizus*, obtained from the firm S.E.A.B.*. This extract contains about 40 lipase units/mg of dry powder. The first step of the purification, a chromatography on Amberlite IRC-50 (ref. 7), is similar to that published by LABOUREUR AND LABROUSSE[9]. Fig. 1 shows that the chromatography on Amberlite IRC-50 (pH 5.7) with elution of the column with an ammonium acetate gradient yields two protein peaks with lipase activity.

The fractions containing lipase I were concentrated by dialysis under vacuum against 1 mM HCl, then 0.33 mM HCl and finally lyophilised. Lipase I obtained by this technique can be stabilised by treatment with 2 mM DFP in 0.5 M Tris–HCl buffer (pH 7.8).

Lipase II is prepared in the absence of DFP from lipase I by allowing it to stand for 7 days at 20° in 50 mM ammonium acetate buffer (pH 5.7) and 5 mM calcium acetate. NaN_3 at a final concentration of 1 mM was added to avoid any bacterial contamination. The mixture was then chromatographed on a column of Amberlite IRC-50 (pH 5.7) under conditions similar to those used for the preparation of lipase I.

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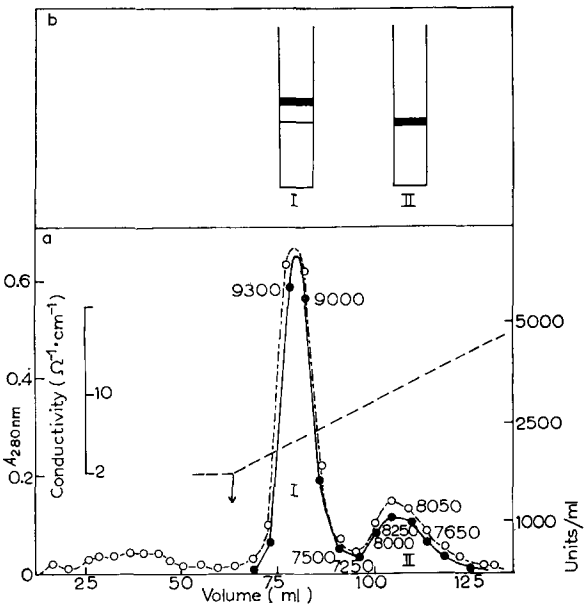

Fig. 1. a. Chromatography on Amberlite IRC-50 of a purified preparation of lipase. Elution with an ammonium acetate gradient. A 0.9 cm × 20 cm column is equilibrated with an ammonium acetate buffer (pH 5.7) containing 5 mM calcium acetate. The lipase sample used comes from a Sephadex G-75 chromatography (see ref. 7). — — —, conductivity of the eluate and as a consequence molarity of the buffer. Arrow shows the gradient front. ○—·—○, absorbance at 280 nm; ●—●, lipase activity (units/ml eluate). Numbers: specific activity of each fraction. b. Disc electrophoresis on polyacrylamide gel, pH 4.3, of lipases I (left) and II (right).

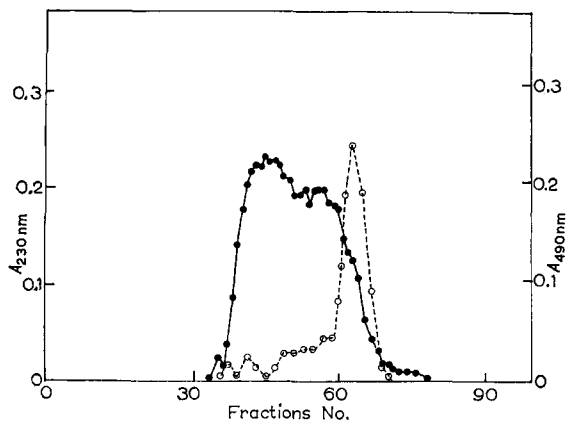

Fig. 2. Separation of peptides and sugars by filtration through Sephadex G-25. The diagram is obtained by chromatography of a total hydrolysate of 8 mg of protein (2 M HCl for 2 h at 100°) through a 1.2 cm × 150 cm column of Sephadex G-25 equilibrated in 0.1 M acetic acid. ○— —○, absorbance at 490 nm obtained after colorimetric titration of sugars; ●—●, absorbance at 230 nm.

The fractions corresponding to the lipase II peak were combined and concentrated by dialysis under vacuum against 1 mM HCl, then 0.33 mM HCl and finally lyophilised.

Detection and identification of sugars

The phenol–H_2SO_4 technique[10] was used for detection of neutral sugars in chromatographic eluates with mannose as the standard. Amino sugars were titrated by the technique of Elson–Morgan, as modified by BOAS[11], and sialic acids were determined by the technique of SVENNERHOLM[12].

Identification of neutral sugars was performed by the following technique. Samples of lipase I (about 8 mg) were hydrolysed in 2 M HCl for 2 h at 100°, in vacuum sealed tubes. The hydrolysate was then filtered through a column (1.2 cm × 150 cm) of Sephadex G-25 equilibrated with 0.1 M acetic acid (Fig. 2). The fractions containing the neutral sugars, located by the phenol–H_2SO_4 mixture, were evaporated. The residue was then chromatographed on Whatman No. 1 paper in the solvent ethyl acetate–pyridine–water (10:4:3, by vol.) (ref. 13). Thin-layer chromatography on Silica gel G impregnated with boric acid in the solvent methylethylketone–acetic acid–methanol (60:20:20, by vol.) (ref. 14) was sometimes used. Sugars were revealed by spraying with a mixture of naphtoresorcinol, 96% ethanol and concentrated H_3PO_4 (respectively 0.2 g, 100 ml, 4.55 ml).

Amino acid analysis and measure of the extinction coefficient

The proteins were hydrolysed with triple-distilled 6 M HCl in sealed tubes at 110° for 24, 48 and 72 h. Amino acids were analysed by the normal technique[15] using a Beckman Spinco 120 B amino acid autoanalyser. Cystine and methionine were titrated following performic acid oxidation in the form of cysteic acid and methionine sulfone, respectively. Tryptophan was determined colorimetrically[16]. The molecular weight, most consistent with the data from the amino acid analysis, was calculated by the technique of DELAAGE[17]. The extinction coefficients calculated from the values based on the amino acid analysis and titration of the sugars are $E^{1\%}_{1\,cm} = 12.5$ for lipase I and $E^{1\%}_{1\,cm} = 11.0$ for lipase II.

RESULTS

Separation of lipase I and II on Amberlite IRC-50 at pH 5.7. Evidence for the presence of carbohydrates in lipase I

Lipase I was partly converted to lipase II by incubation at 22° for 4 days. The mixture obtained was chromatographed on a 1.7 cm × 27 cm column of Amberlite IRC-50 at pH 5.7 under ordinary conditions. Neutral sugars, proteins and enzyme activity were determined in each fraction. Fig. 3 shows that lipase I contains large amounts of neutral sugars. Lipase II, on the other hand, is practically devoid of them. In addition, the transformation of lipase I into lipase II parallels the appearance of a fraction (A) which is not retarded on the Amberlite IRC-50 column. This fraction contains the neutral sugars, no longer a part of lipase II, and also protein and peptide components which absorb slightly at 280 nm and can be measured by the colorimetric technique of LOWRY *et al.*[8]. This experiment can be compared with another, recently described by LABOUREUR AND LABROUSSE[18], leading to similar conclusions. Lipase I therefore changes to lipase II with the loss of almost all its carbohydrates. Further-

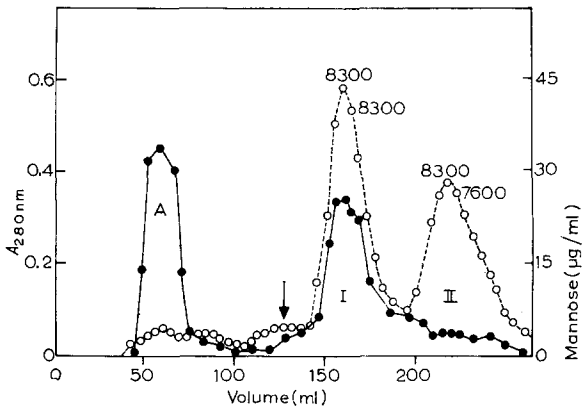

Fig. 3. Determination of neutral sugars in the chromatographic eluate of a mixture of lipases I and II. Lipase I was stored at 22° in a buffer (pH 5.7) for 4 days before chromatography on a 1.7 cm × 27 cm column of Amberlite IRC-50. Elution is carried out as described in Fig. 1. ○— — —○, absorbance at 280 nm; ●—●, μg of mannose per ml of eluate. Numbers indicate the specific activity of the fractions. The arrow shows the gradient front.

TABLE I

AMINO ACID COMPOSITION OF LIPASE I, OF LIPASE I AFTER SEPARATION OF THE GLYCOPROTEIN FRACTION AND OF GLYCOPROTEIN FRACTION

Amino acid	*Number of residues in 1 mole of*						
	Lipase I		*Lipase I after separation of the glycoprotein fraction*		*Glycoprotein fraction*		*Glycoprotein fraction + precipitated lipase I*
	Experimental value	*Nearest whole number*	*Experimental value*	*Nearest whole number*	*Experimental value*	*Nearest whole number*	*Integral value*
Ala	23.4	23	18.66	19	5.00	5	24
Arg	10.08	10	8.72	9	0.98	1	10
Asx	39.80	40	28.67	29	7.67	8	37
Cys	5.93	6	5.30	5	1.03	1	6
Glx	29.50	29–30	26.26	26	5.54	5–6	31–32
Gly	28.08	28	25.65	26	4.45	4–5	30–31
His	8.38	8	7.15	7	1.06	1	8
Ile	19.67	20	17.97	18	2.04	2	20
Leu	24.54	24–25	19.49	19–20	4.40	4–5	24–25
Lys	20.04	20	16.17	16	4.18	4	20
Met	2.94	3	0.92	1	0.80	1	2
Phe	15.25	15	16.93	17	0	0	17
Pro	23.71	24	16.77	17	5.25	5	22
Ser	40.00	40	30.59	31	8.72	9	40
Thr	28.17	28	27.79	28	2.58	2–3	30–31
Trp	6.00	6					
Tyr	14.63	15.0	14.05	14	1.09	1	15
Val	28.96	29	28.37	28	0.94	1	29
Mol. wt.	40 281 ± 750		34 270 ± 1000		6100 ± 300		

more, since the specific activity of lipase II is of the same magnitude as that of lipase I, the sugars are apparently not necessary for the catalytic capacity of the enzyme.

Molecular properties of lipase I

The molecular weight of lipase I as estimated by filtration through a Sephadex G-100 column was 40 000 ± 2000 (ref. 7). Table I shows the amino acid composition of the enzyme.

The molecular weight most consistent with this analysis, as calculated by the method of DELAAGE[17], was 40 250 ± 750 for the protein portion of lipase I.

The identification of the neutral sugars in lipase I was carried out by the techniques described in MATERIALS AND METHODS. It was found that one molecule of lipase I contains 13–14 molecules of mannose. It was also possible to observe the kinetics of the liberation of amino sugars by hydrolysing about 10 mg of lipase with 4 M HCl at 110° for 4, 6 and 9 h. The hydrolysates were chromatographed in a microcolumn of Dowex 50 X-4. Amino sugars were eluted with 1 M HCl and separated from interfering products according to Elson and Morgan[11]. The results indicated the presence of 1.4, 1.6 and 1.53 moles of total amino sugars per mole of lipase after 4, 6 and 9 h of hydrolysis, respectively. The maximum value of 1.6 mole of hexosamine per mole of enzyme found after 6-h hydrolysis was retained, and it was considered that one molecule of lipase I contains 2 molecules of hexosamine, the exact nature of which is not yet known precisely. It was not possible to demonstrate the presence of sialic acids by the technique of SVENNERHOLM[12].

The conclusion of these assays is that each molecule of lipase I is associated with 13–14 molecules of mannose and 2 molecules of hexosamine. This corresponds to 2500 g of total carbohydrate per mole of lipase I, which results in a total molecular weight of 42 750. This value is consistent with that previously determined by Sephadex G-100 filtration.

In parallel with the studies on the carbohydrate portion of lipase I, studies on the protein portion were undertaken. The technique of Sanger showed the presence of an N-terminal aspartic acid (or asparagine) residue in the samples treated with DFP (Table II). Nontreated lipase revealed, in addition to the aspartic acid residue, signi-

TABLE II

N-TERMINAL ENDS OF LIPASES I AND II

The results, expressed in moles of DNP-amino acids per mole of lipase, are corrected to compensate losses during hydrolysis, sublimation and paper chromatography. These losses are estimated to 50% for the normal amino acids (Asp, Glu, Ala, Val) and 70% for DNP-Ser and DNP-Thr. The absorption of a millimolar solution of these amino acids is taken as equal to 16.2. The molecular weights of DNP-proteins are calculated from their amino acid composition to which is added the contribution of the fixed DNP-radical.

Lipase	*DNP-amino acids (moles/mole lipase I)**					
	Asp	*Thr*	*Ser*	*Glu*	*Val*	*Ala*
Lipase I treated with DFP	0.63					
Lipase I untreated with DFP	0.62		0.16			0.11
Lipase II	0.006	0.17	0.36	0.06	0.01	0.06

* Corrected values.

ficant amounts of N-terminal alanine and serine. These results do not agree with those recently published by LABOUREUR AND LABROUSSE[18] who indicate that the lipase of *Rhizopus arrhizus* contains a serine as N-terminal residue. In our assays, 0.3 mole/mole of N-terminal serine was also identified in a sample of lipase I untreated with DFP. This serine, which was the single detectable N-terminal residue, probably arises from a partial degradation of the enzyme.

Mode of binding of the sugars to the protein. Existence of a glycoprotein fraction bound to lipase I

To ascertain the nature of the bonds between the sugars and the protein, three different techniques were used: addition of 5% trichloroacetic acid to the solution of lipase I at 0°; heating of the solution in a boiling-water bath for 3 min and addition of 6 M HCl in the cold until the pH dropped below 1.0. All these techniques gave similar results, yielding a precipitate and a supernatant solution whose characteristics are given in Table III, in the case of trichloroacetic acid treatment. The data show that the sugar–protein bond is almost completely cleaved. The precipitate consists largely of the denaturated protein, while the detached sugar (or sugars) is soluble. It would appear, therefore, that the sugars are not covalently bound to lipase.

However, a sizeable amount of protein material, absorbing at 280 nm, is also present in the solution. This is due, as shown later, to the existence of a glycoprotein complex designated as the "glycoprotein fraction".

Characterisation of the protein fraction of precipitated lipase I

Following precipitation at pH below 1.0, the protein precipitate obtained after centrifugation is soluble in distilled water. But the solution is no longer enzymatically active. One can however analyse the protein separated from the glycoprotein fraction. The results in Table I show that lipase I has lost about 60 amino acids during the precipitation procedure, among which 2 methionine residues out of 3, 1 histidine,

TABLE III

SUPERNATANT COMPOSITIONS AFTER PRECIPITATION WITH 5% TRICHLOROACETIC ACID AT 0° OF SOLUTIONS (1–1.5 mg/ml) OF LIPASE I

Expt. No.	*% of the absorbance at 280 nm found in the supernatant (proteins)*	*% of the absorbance at 490 nm found in the supernatant after titration of sugars with the phenol–H_2SO_4 technique*
1	9.5	86.5
2	8.4	87.5
3	11.5	84
4	4.0	88
5	6.4	85.5
6	1.3	80
7	2.2	75

1 tyrosine and 1 half-cystine as well as several aspartic acid and serine residues. On the other hand, no phenylalanine residues seem to be lost.

It should be noted that precipitation of lipase I and simultaneous elimination of the glycoprotein fraction does not modify the N-terminal end of the molecule, which is still aspartic acid (or asparagine).

Characterisation of the glycoprotein fraction

After lyophilisation of the supernatant, the glycoprotein fraction is filtered through a column of Sephadex G-50 "fine" equilibrated with 0.15 M pyridine–acetate buffer (pH 5.5). The elution diagram is reproduced in Fig. 4. The greatest part of the carbohydrate and protein material is found under a single peak eluted with the first void volume. This is consistent with a molecular weight of at least 7000–8000 for the apparently unique component of the glycoprotein fraction.

This fraction was also studied by disc electrophoresis on 15% polyacrylamide gel (pH 8.6). A single band, slightly anionic at this pH, was observed. The analysis of the cationic gel did not reveal the presence of any other component. So it appears that the glycoprotein fraction was composed of a single glycopeptide component. Its sugar composition was similar to that of lipase I (13–14 molecules of mannose and 2 molecules of amino sugar).

Table I also shows the amino acid composition of the glycoprotein fraction. This composition cannot be taken as definite, since the presence of sugars can lead to anomalous results[19]. The molecular weight of the peptide part of the glycoprotein

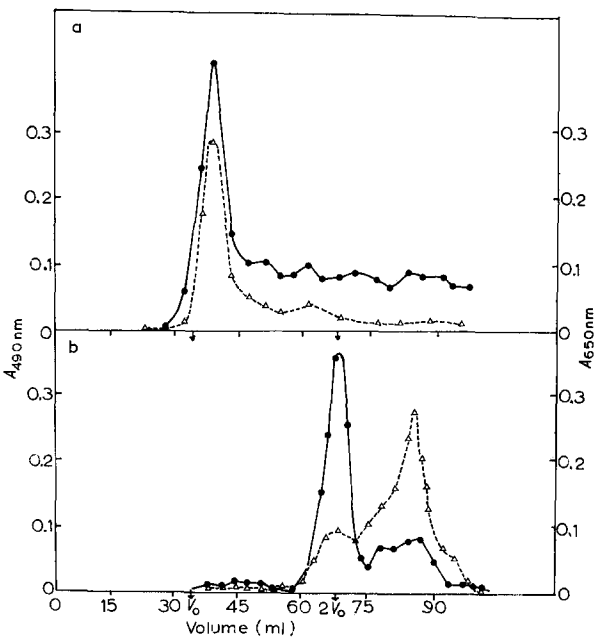

Fig. 4. a. Filtration through Sephadex G-50 of the glycoprotein fraction separated from lipase I. b. Filtration through Sephadex G-50 of Fraction A. The column (0.9 cm × 150 cm) of Sephadex G-50 "fine" is equilibrated in 0.15 M pyridine–acetate buffer (pH 5.5). ●—●, absorbance at 490 nm (titration of sugars); △— — —△, absorbance at 650 nm (titration by the Lowry technique).

fraction calculated from these results is 6100. The total molecular weight of the glycoprotein fraction is then about 8600 (2500 for the sugar fraction and 6100 for the peptide fraction). It is worthwhile noting that the peptide part contains a single half-cystine residue titrated in the form of cysteic acid, 1 histidine residue, 1 arginine and 1 tyrosine. Phenylalanine is not present. The presence of tryptophan is uncertain considering the difficulties encountered in the determination of this amino acid. If one adds the amino acid residues of the glycoprotein fraction to those of precipitated lipase I, one finds almost the same composition as for native lipase I (Table I), except for methionine which is probably destroyed during hydrolysis. On the whole, the results obtained are consistent with the idea that a glycopeptide of molecular weight around 8600 is bound to the Rhizopus lipase by noncovalent bonds.

Molecular properties of lipase II

The "spontaneous" transformation of lipase I into lipase II induces the appearance of a glycopeptide fraction similar to the glycoprotein fraction, which we have called Fraction A. The difference between the molecular weights of lipases I and II was estimated to be around 10 000, and lipase II no longer contains the sugars and some of the amino acids present in lipase I. In particular it is devoid of amino sugars and contains less than 1 mole of mannose per mole of enzyme.

The amino acid composition of lipase II is shown in Table IV. The molecular weight consistent with this composition is about 32 000 which is in good accordance with the value found with the Sephadex technique (30 000 ± 1500). The compositions

TABLE IV

AMINO ACID COMPOSITION OF LIPASE II

Amino acid	*Number of residues in 1 mole of*			
	Lipase II		*Lipase I after separation of the glycoprotein fraction*	
	Experimental value	*Nearest whole number*	*Experimental value*	*Nearest whole number*
Ala	17.69	18	18.66	19
Arg	8.98	9	8.72	9
Asx	27.51	27–28	28.67	29
Cys	5.45	5	5.30	5
Glx	25.07	25	26.26	26
Gly	23.48	23–24	25.65	25–26
His	6.95	7	7.15	7
Ile	18.11	18	17.97	18
Leu	18.03	18	19.49	19–20
Lys	15.44	15–16	16.17	16
Met	1.05	1	0.92	1
Phe	15.18	15	16.93	17
Pro	16.45	16–17	16.77	17
Ser	26.9	27	30.59	30–31
Thr	23.9	24	27.79	28
Trp	3.55	4		
Tyr	11.99	12	14.05	14
Val	28.54	28–29	28.37	28
Mol. wt.	32 128 ± 800		34 270 ± 1000	

of lipase II and of the precipitated lipase I are very similar. However lipase II seems to be slightly smaller (molecular weight 32 000 instead of 34 000) and contains about 20 amino acids fewer (Table IV).

Moreover its N-terminal end is no longer aspartic acid which has been replaced, as shown in Table II, by several other residues present in small quantities. Among these new residues, serine, threonine, alanine and valine are found. It seems thus that lipase II is a degraded form of lipase I. It seems to be distinguishable from it not only as with the precipitated lipase by the removal of the glycopeptide fractions but also by a significant degradation of the enzyme itself. This last degradation must however be slight, since lipase II remains homogeneous by disc electrophoresis and also preserves nearly all of its enzymatic activity.

Characterization of the Fraction A

It was of interest to determine the difference between the A and glycoprotein fractions. Fraction A was collected, lyophilised and analysed. The number of μmoles of Fraction A analysed was calculated from its neutral sugar content. Since lipase I contains 2200 g of mannose per mole, the number of moles of lipase I transformed into lipase II can be deduced from the amount of neutral sugars found in Fraction A. The results obtained are indicated in Table V and compared with those obtained for the glycoprotein fraction. The reproducibility of three analyses carried out on three independently prepared samples of Fraction A was good and suggests that Fraction A contains more amino acids than does the glycoprotein fraction, the most striking difference being the presence of one phenylalanine residue which is absent from the

TABLE V

AMINO ACID COMPOSITION OF FRACTION A

Amino acid	*Number of residues per mole of lipase I transformed, included in Fraction A*				*Number of residues in*		
	1	*2*	*3*	*Mean*	*Glycoprotein fraction*	*Lipase II + Fraction A*	*Lipase I*
Ala	4	6	6	5–6	5	23–24	23
Arg	0	0	1	0–1	1	9–10	10
Asx	9	11	12	11	8	38–39	40
Cys					1		6
Glx	5	5	5–6	5	5–6	30	29–30
Gly	6	6	5	6	4–5	29–30	28
His	0	0	0–1	0–1	1	7–8	8
Ile	3	2–3	3	3	2	21	20
Leu	7–8	7	7–8	7–8	4–5	25–26	24–25
Lys	0	1	1	1	4	16–17	20
Met	1	1	1	1	1	2	3
Phe	1	1	1	1	0	16	15
Pro	5–6	6	6	6	5	22–23	24
Ser	8	11	8–9	9	9	36	40
Thr	5	4	5	4–5	2–3	28–29	28
Tyr	2–3	2	2	2	1	14	15
Val	2–3	2–3	3	3	1	29–30	29
Total amino acids				62–67	54–58		

glycoprotein fraction. The amount of basic residues is, on the other hand, somewhat smaller.

The sugar composition of Fraction A is similar to that of lipase I. Qualitatively it appears that A and glycoprotein fractions are similar. However, we know that the glycoprotein fraction behaves as a single component during chromatography through Sephadex G-50 (Fig. 4). On the other hand, Fraction A is certainly heterogeneous (Fig. 3). The elution diagram obtained after filtration of this fraction through a 0.9 cm × 150 cm column of Sephadex G-50 "fine" equilibrated with 0.15 M pyridine–acetate (pH 5.5) is shown in Fig. 4. If this diagram is compared with the one obtained with the glycoprotein fraction, it can be seen that nothing is eluted before 2 void vol. of the column. At the beginning of the 3rd void vol., a fraction is eluted which is rich in neutral sugars (phenol–H_2SO_4 titration) but contains very little peptide material analysable by the method of LOWRY *et al.*[8]. This fraction appears before a second very heterogeneous peak, Lowry positive, but containing very little neutral sugar. Since these materials are eluted nearly at the same time as inorganic salts, their molecular weight is probably relatively low. The presence in Fraction A of a number of short peptides can also be shown by chromatography in a Technicon autoanalyser.

All these observations taken together suggest that Fraction A, obtained during long storage of lipase I, is a mixture of peptides and glycopeptides arising from the degradation of the glycoprotein fraction. This degradation seems to promote spontaneous cleavage of the bond between the glycopeptide and the lipase.

DISCUSSION

This work shows that the mold *Rhizopus arrhizus* synthetises a single exocellular lipase, a glycoprotein of molecular weight around 43 000 containing 2200 g of mannose and 340 g of amino sugar per mole. This confirms the study of LABOUREUR AND LABROUSSE[18] who have also recently shown the glycoproteic nature of this enzyme. The presence of sugars in lipases from other microorganisms has been demonstrated before[1,2]. Their possible role in the enzymic activity, however, was not defined until now. The present work shows that, while the lipase of *Rhizopus arrhizus* contains a mixed glycopeptide complex, this complex does not play any role in the activity of the enzyme. Indeed, lipase II, nearly devoid of sugar, is nearly as active as lipase I. This is analogous to the finding that bovine ribonuclease A devoid of sugars is as active as ribonuclease B which contains an appreciable amount of sugars[20].

However, it would appear that lipase I is not a real glycoprotein in the classical meaning of the term since the sugars are not covalently bound to the major part of protein. This conclusion is based upon the finding that it is possible, to separate from the enzyme a glycoprotein fraction with certain denaturing agents. This glycoprotein fraction is a unique component, homogeneous by disc electrophoresis, with a molecular weight of about 8500. Its high solubility at elevated temperature (100°), in acid medium and in 5% trichloroacetic acid is certainly caused by its high sugar content which represents more than 25% of the total weight of the glycoprotein molecule.

It is very likely that the methods used for the separation of the glycoprotein fraction from the remainder of the enzyme are not able to break covalent bonds under the conditions employed. These agents probably are able to weaken the slight inter-

actions between the glycoprotein fraction and the protein. However, since little is, in fact, known about the stability of glycosidic bonds, it is not completely excluded that particularly labile bonds of this type are broken by cold 5% trichloroacetic acid, upon short heating to 100° or by cold HCl at pH 0.8.

It should be noted that classical denaturing agents, such as urea, are unable to dissociate the glycopeptide fraction from lipase I. It was also not possible to dissociate the glycoprotein fraction under conditions in which the lipase activity was preserved. Thus, $(NH_4)_2SO_4$ precipitation or chromatography in a column of Biogel P-100 equilibrated at different pH's (pH 3.0, 5.5 and 9.0) do not destroy the association. Apparently lipase must be irreversibly denatured for the cleavage to occur.

While this glycoprotein portion apparently plays no role in the catalytic activity, another role can be assigned to it which involves the hypothesis of EYLAR[21] according to which the temporary association of a protein with a sugar could facilitate the passage of the protein through membranes. The lipase of *Rhizopus arrhizus* is exocellular and it may be that the role of the carbohydrate portion is to aid in its crossing the bacterial membrane. It is conceivable that those few glycoproteins encountered in other secretory organs such as the pancreas are vestiges of a distant era during which the system of zymogen granules was not in use and the pancreatic enzymes also were obliged to cross membranes.

The compositions of the A and glycoprotein fractions are sufficiently similar to suggest that the slow transformation of lipase I into lipase II occurs in the same manner as the rapid dissociation of lipase from the glycoprotein fraction with 5% trichloroacetic acid. In both cases a glycoprotein fraction is separated from the lipase. However, storage of lipase I promotes degradations. The stabilizing effect of DFP upon the transformation of lipase I into lipase II suggests that this process is enzymatic. Our preparations could be contaminated with small amounts of one or several enzymes whose presence is not detectable by disc electrophoresis. The fact that peptides are liberated and new N-terminal residues appear during the transformation allow one to consider the intervention of a proteolytic enzyme. But it is not possible to be more precise, considering our very limited knowledge of the type of bond broken during the transformation.

The separation of the enzyme protein from the glycoprotein fraction, which occurs spontaneously in the presence of trichloroacetic acid or high temperature, seems to require at least a beginning of degradation during the transformation of lipase I into lipase II. Indeed, it may be that noncovalent bonds are stable as long as the glycoprotein fraction is intact and that they are weakened by its degradation. Some bonds may be essential to maintain the conformation required for the interaction between the enzyme and the glycoprotein fraction.

Lipase II seems to have been degraded somewhat since for a single N-terminal aspartic acid residue have been substituted several end groups in non-stoichiometric amounts. We have not studied the C-terminal end. One may derive a very preliminary indication of the amount of degradation of lipase II from a comparison of its composition with that of lipase I separated from the glycoprotein fraction (Table IV) or by comparing Fraction A to the glycoprotein fraction (Table V). These data seem to indicate that, in addition to the glycoprotein fraction, about 20 amino acids have been split off during the transformation of lipase I into lipase II. It is thus possible to obtain a partially degraded lipase which still has almost full catalytic activity.

It would be very interesting to compare the molecular and catalytic properties of lipase II with an intact active lipase freed from its glycoprotein fraction.

Finally it seems interesting to point out that this lipase II contains an odd number (5) of half-cystine residues, since a half-cystine is separated during the transformation of lipase I into lipase II and is found in the glycoprotein fraction.

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