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HYDROLYSIS OF LONG-CHAIN MONOGLYCERIDES IN MICELLAR SOLUTION BY PANCREATIC LIPASE

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SUMMARY

1. Certain long-chain monoglycerides have a high solubility in bile-salt solutions forming mixed monoglyceride–bile-salt micelles. Monoglycerides in such an optically clear, micellar solution are readily hydrolyzed by pancreatic lipase (glycerol ester hydrolase, EC 3.1.1.3). Using a rat-pancreatic-juice preparation containing negligible esterase activity, the hydrolysis of a number of pure monoglycerides in micellar solution has been studied.

2. The rate of hydrolysis was essentially linear with time and enzyme concentration up to a level of around 60–70 % hydrolysis of the substrate. The extent of hydrolysis fell when the concentration of micelles of identical composition was increased. There was a broad pH optimum 5.5–7.5.

Bile salts greatly enhanced the rate of hydrolysis at low concentrations, but this effect was possibly chiefly due to their dispersing effect; at higher concentrations, they caused a marked inhibition of hydrolysis. Typical anionic detergents influenced hydrolysis in the same manner as bile salts. With a cationic detergent the enzyme concentration had to be considerably increased to obtain the same rate of hydrolysis.

3. 2-Monoolein was not attacked to any appreciable extent by pancreatic lipase; thus the specificity of lipase for the 1-ester position could be directly shown. Glycol monooleate was readily hydrolyzed; 1-α,α-dimethyl-monodecanoin was not split.

4. Under experimental conditions where 1-monoolein was extensively hydrolyzed, middle-chain saturated 1-monoglycerides were hydrolyzed to a very limited extent. Extensive hydrolysis could be obtained by increasing the micellar amphiphile to micellar bile-salt ratio, by adding other amphiphiles such as fatty acid, or by using a two-phase heptane–buffer system where the monoglyceride was concentrated at the interface. The results of these and other experiments were interpreted as showing the tightness of packing of the micelle to be a critical factor influencing the extent of hydrolysis.

5. Titration of the fatty acid of a mixed bile salt–monoglyceride–fatty acid micelle indicated a pK_a of 6.9 with the experimental conditions present.

Abbreviation: CMC, critical micellar concentration.

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INTRODUCTION

Substrates for pancreatic lipase (glycerol ester hydrolase EC 3.1.1.3) are generally prepared in the form of an emulsion, and recently evidence has been presented that pancreatic lipase will attack only emulsified substrates[1]. It is possible, however, to disperse lipids in aqueous solution in another type of aggregate, namely a mixed micelle, if the proper detergent is used[2]. Such optically clear, micellar solutions are completely stable. The concentration of lipid obtained is usually much lower than in a typical emulsion, and the ratio of detergent or amphipath to lipid is far higher than in an emulsion, where the amphipath is restricted to the oil–water interface[3] and composes but a minor fraction of the particle. A great deal of work has been done on the properties of micellar solutions, and substrates in such form can be considered to be rather well-defined with respect to molecular arrangement, at least when paraffin chain ionic detergents are used as the amphipath*,[4–6].

Some time ago, it was observed in this laboratory that certain monoglycerides had a high solubility in bile-salt solution[13]. They were solubilized as amphiphiles (for definition see HOFMANN[14]) with saturation ratios (micellar monoglyceride/micellar bile salt) approaching 2. Monoglycerides in such optically clear solutions have now been found to be hydrolyzed readily by pancreatic lipase. This paper presents studies on the action of pancreatic lipase on a number of pure monoglycerides, the substrate being in the form of a mixed monoglyceride-bile salt micelle. Although these experiments were undertaken with the hope that the use of micellar substrates would simplify the study of pancreatic lipase, the reverse has proved to be the case. As pointed out by DESNUELLE[1] the usual terms of enzymology are inadequate for the description of such systems.

MATERIALS AND METHODS

Bile salts were prepared by conjugation of purified cholic or deoxycholic acid according to NORMAN[15] as described[16]. They were at least 97% pure by thin-layer chromatography, the chief impurity being unconjugated acid. Bile-salt solutions were prepared which were 24 mM in bile salt and 126 mM in NaCl; they were thus 0.15 M in Na^+. Typical anionic detergents, sodium lauryl sulfate, sodium oleyl taurate, and sodium *p*-(*n*-octylbenzene) sulfonate of high purity were used; they have been characterized elsewhere[14]. As cationic detergent cetyl-trimethyl ammonium bromide (T. Schuchardt, Munich, Germany) was used. Solutions, 0.15 M in Na^+ were prepared as described for bile salts. Buffers, 0.15 M in Na^+ were prepared as described[14].

Pancreatic lipase

As a source of pancreatic lipase pancreatic juice, obtained from rats by cannu-

* BANGHAM AND DAWSON[7–11], in their studies on phospholipases used phospholipid dispersed in buffer, which, according to these workers, was probably present as laminated structures consisting of bimolecular layers of phospholipid molecules with their hydrophilic portions adjacent and separated by a layer of water molecules. They used the term "emulsion" or "micelle" to describe these dispersions. We have used the term "micelle" as originally defined[12], *i.e.*, a spherical aggregate of detergent molecules, which contains a liquid hydrocarbon interior and is in equilibrium with the unassociated detergent molecules. We think it is reasonable and helpful to distinguish, whenever possible, such a micellar state of aggregation from a dispersion of myelin figures and each of these, moreover, from an oil-in-water emulsion.

lation, was used. The pancreatic juice was collected over ice and lyophilized. Preparations thus prepared could be stored without loss of activity for months. Lyophilized preparations of rat-pancreatic juice do not show any esterase activity[17]. In general, the lipase preparation was dissolved in the 0.15 M buffer (pH 6.3) to a volume corresponding to 10 times the original juice volume; the solution was prepared shortly before the experiment. The pancreatic juice solution could be incubated for 1 h at 37° without loss of activity.

1- and 2-monoglycerides and structural analogues have been described[18]. Fatty acids and alkyl alcohols were commercial samples, generally Eastman Kodak (Rochester, New York). They were not characterized with respect to purity, as homologue purity was not essential to the experiments in which they were used.

Incubations

Monoglyceride or other lipid was added in the appropriate solvent, generally heptane or chloroform, to sterile glass 10-ml ampoules, and the solvent removed by evaporation. Buffer and bile salt solution were added, and the ampoules shaken at 37° until the lipid was completely dissolved, or dispersed if the amount added was greater than its solubility. The appropriate aliquot of pancreatic lipase was added to a total volume of 2.0 ml. The ampoules tips were then fused in a gas–oxygen flame and the ampoules were incubated 1 h, the ampoules were inspected, their contents transferred to glass stoppered test tubes, and the lipid extracted as described[18].

The pooled upper phases were evaporated, and the residue dissolved in a small volume of ethanol and titrated under CO_2 free air with 0.02 N methanolic NaOH. Control experiments had shown quantitative recovery of fatty acids from bile salt or bile salt–monoglyceride solution at pH 6.3 with no transfer of bile acids to the upper phase.

The majority of experiments were performed at pH 6.3 with bile salt and monoglyceride concentrations of 6–12 μmoles/ml and at 37°. The Na^+ concentration was 0.15 M in all experiments.

For experiments concerned with the influence of pH, ampoules were prepared containing identical amounts of monoglyceride and bile salt, with buffers of differing pH, but all buffers being 0.15 M in Na^+. Controls without lipase were run at each pH.

Boiled enzyme controls were not run, but the amount of titratable material when lipase was omitted, when monoglyceride was omitted, or when a non-hydrolyzable monoglyceride was used was negligible. All experiments were run in duplicate with excellent agreement. In experiments performed with added fatty acid, identical aliquots of the fatty acid solutions used were titrated directly, and the experimental results corrected accordingly. In some experiments, the reaction products were separated by thin-layer chromatography on silicic acid[19].

In order to estimate the degree of ionization of the fatty acid formed in the mixed micelle, titration of oleic acid in a sodium taurodeoxycholate–monoolein–oleic acid micellar solution was performed. Titration was performed at room temperature, the solution remaining clear throughout the titration. The micellar solution was acidified with 1 M HCl (to pH 2) then titrated with 0.05 N NaOH. Control solutions containing bile salt and monoolein but no oleic acid were titrated and the resultant curve subtracted from that obtained with the micellar solutions containing oleic acid. The concentration of bile salt and monoglyceride employed were the same as those

in the hydrolysis experiments; the Na^+ concentration in both the micellar solution titrated and the NaOH was 0.15 M. Because of the influence of the ionized fatty acid on the remaining unionized fatty acid, the titration curves obtained were not symmetrical. Therefore, the pH corresponding to half neutralization was taken as the pK_a'.

RESULTS

Results are shown in per cent hydrolysis and in some cases are supplemented by fatty acid liberated, expressed in μequiv/ml incubation mixture. Experiments were conducted for 1 h; the term "extent of hydrolysis" means the percentage hydrolysis of substrate in that time.

Previous studies have defined the behavior of 1-monoolein[14] and other monoglycerides[18] in dilute bile salt solutions. In sodium taurodeoxycholate, the CMC with 1-monoolein as the solute is about 0.8 mM (see ref. 14). The concentration of micellar bile salt is the total bile-salt concentration minus 0.8. The concentration of micellar 1-monoolein equals the total 1-monoolein concentration as all 1-monoolein present should be in micellar form. Therefore for any concentration of bile salt and 1-monoolein, indeed for any bile salt of which the CMC is known with 1-monoolein as solute, the micellar composition is easily calculated. This is most conveniently expressed as the ratio of micellar monoolein or amphiphile to micellar bile salt or detergent and such a value will be termed the A_m/D_m ratio. The A_m/D_m ratio will be less than the saturation ratio which is the A_m/D_m ratio when the micelle is saturated with amphiphile[14].

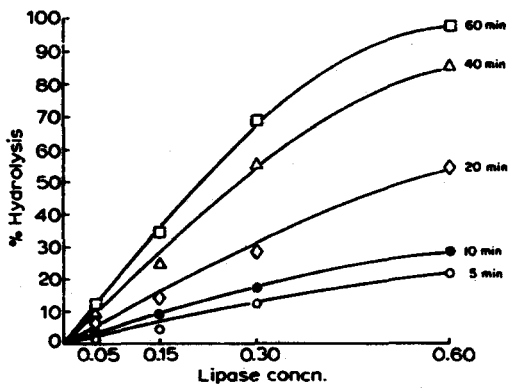

Fig. 1. Effect of pancreatic lipase concentration (in arbitrary volume units) on hydrolysis of micellar 1-monoolein. The extent of hydrolysis was determined at different time intervals, as shown. 1-Monoolein, 12 μmoles/ml; sodium taurodeoxycholate, 12 μmoles/ml. Sodium phosphate buffer (pH 6.3) and 0.15 M in Na^+ was used at 37°. Volume of incubation mixture, 2.0 ml.

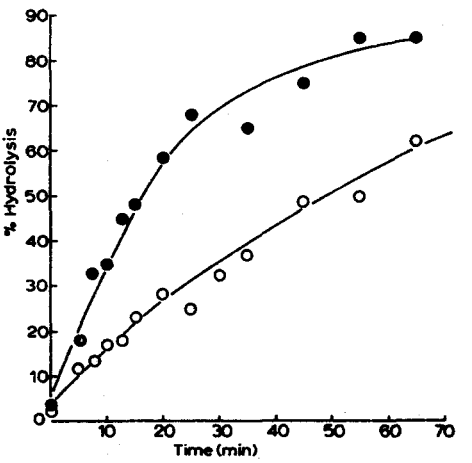

Fig. 2. Time course of hydrolysis of micellar 1-monoolein by pancreatic lipase. Upper curve (●—●): 1-monoolein, 6 μmoles/ml; sodium glycodeoxycholate, 7.2 μmoles/ml; 0.2 ml lipase. Lower curve (○—○): 1-monoolein, 6 μmoles/ml; sodium glycodeoxycholate, 13.2 μmoles/ml; 0.8 ml lipase. Experimental conditions otherwise as in Fig. 1. The slower rate of hydrolysis of the lower curve, despite four times as much enzyme being present, reflects the lower A_m/D_m ratio (see text).

Effect of enzyme concentration

Fig. 1 shows that the percentage hydrolysis was proportional to the enzyme concentration for 1 monoolein up to an extent of hydrolysis of 60–70 %.

Time course of hydrolysis

In Fig. 2 is graphed the percentage hydrolysis *versus* time at two different enzyme levels. The hydrolysis appears to be approximately linear with time, at least for the first portion of the curve.

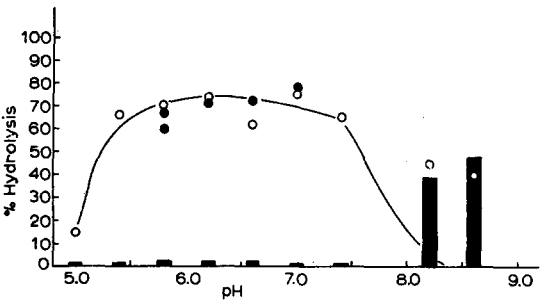

Fig. 3. Effect of pH on extent of hydrolysis of micellar 1-monoolein by pancreatic lipase. In both, 1-monoolein, 12 μmoles/ml; ●—●, sodium glycodeoxycholate (10 μmoles/ml); O—O, sodium taurodeoxycholate (10 μmoles/ml). The black bars represent hydrolysis occurring in the control ampoules prepared without lipase, *i.e.*, non-enzymic hydrolysis. The continuous line is the difference between total hydrolysis and non-enzymic hydrolysis and represents enzymic hydrolysis. Experimental conditions other than pH as in Fig. 1; duration of experiment, 1 h.

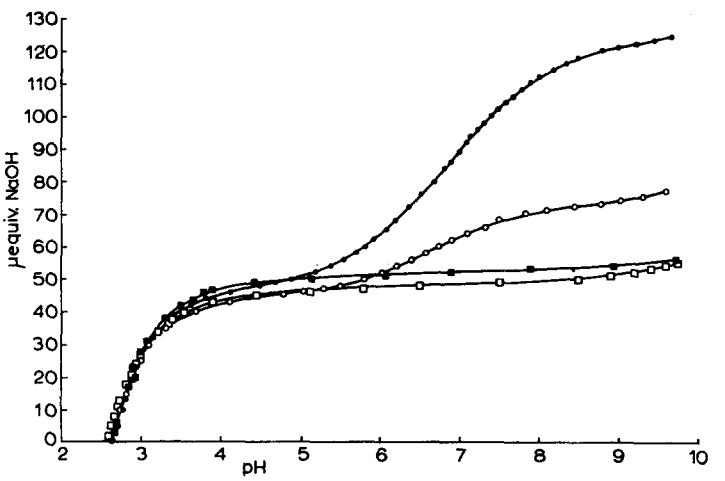

Fig. 4. Titration curves of oleic acid in 1-monoolein–oleic acid–sodium taurodeoxycholate micelles. See METHODS. 1-Monoolein, 1.6 μmoles/ml; oleic acid, 4.8 μmoles/ml; sodium taurodeoxycholate, 6 μmoles/ml (●—●). 1-Monoolein, 4.8 μmoles/ml; oleic acid, 1.6 μmole/ml; sodium taurodeoxycholate, 6 μmoles/ml (O—O). Control titration of 1-monoolein, 4.8 μmoles/ml in sodium taurodeoxycholate. 6 μmoles/ml (■—■). Control titration of sodium taurodeoxycholate alone, 6 μmoles/ml (□—□). In the titration shown in the upper curve, 72 μmoles fatty acid were titrated; in the curve under this, 16 μmoles. The pH corresponding to half neutralization in both curves is about 6.9, but the curves are neither symmetrical nor superimposable. At pH 6.3, a slightly greater fraction of the fatty acid in the solution with the higher fatty acid to monoglyceride ratio is ionized.

Effect of pH

There was a broad pH optimum from pH 5.5 to pH 7.5 (Fig. 3). Above pH 8, spontaneous hydrolysis of micellar 1-monoolein began. Titration experiments, shown in Fig. 4, indicate the pK_a' of oleic acid in the mixed bile salt–monoolein–oleic acid micelle to be 6.9. The pK_a' was not significantly affected by the ratio of monoolein to oleic acid in the micelle, but the shape of the titration curve was to some extent (see legend). Thus, at pH 6.3, as the hydrolysis progresses, a greater percentage of the liberated fatty acid in the micelle is ionized. The charge of the micelle increases more rapidly than the number of ester bonds split. Other titration experiments, to be reported, revealed the pK_a' of oleic acid in monoolein–oleic acid–bile salt micelles at higher bile salt concentrations to fall to values approaching 6.1.

Effect of bile salt concentration

The effect of sodium glycodeoxycholate and sodium taurodeoxycholate on the extent of hydrolysis is shown in Fig. 5. There was a marked increase in the hydrolysis with a low concentration of bile salts. However, with higher bile salt concentrations, there was increasing inhibition. Fig. 6 shows the same experiment with the trihydroxy-bile salts, sodium glycocholate and sodium taurocholate. These bile acids have a higher CMC (see refs. 14, 20) and it is evident that extensive hydrolysis occurs below their CMC. The same enhancement of hydrolysis at low concentrations and inhibition at high concentrations was observed with the typical anionic detergent sodium *p*-(*n*-octylbenzene) sulfonate. Thus, the bile salt effect does not seem to be specific. Nor were there any differences in the effect of sodium tauro- or glyco-cholate, or sodium taurodeoxy- or glycodeoxycholate, when the higher CMC of the trihydroxy conjugates was taken into account (see legend to Fig. 6).

Above the CMC, added bile salt anions are entirely incorporated into the micelles[21]; therefore, the concentration of unassociated bile salt anions remains constant. The addition of bile salt accordingly results in a change in the A_m/D_m ratio and the inhibition observed at higher bile salt concentration must be considered with regard to changes in the micellar composition. Inhibition occurred when the A_m/D_m

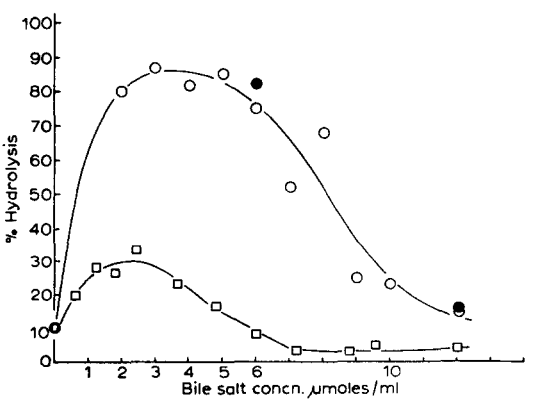

Fig. 5. Effect of sodium glycodeoxycholate (○—○, □—□), or sodium taurodeoxycholate (●—●) on hydrolysis of micellar 1-monoolein. In all experiments, 1-monoolein, 6 μmoles/ml. Upper curve (○—○, ●—●) 0.2 ml enzyme; lower curve (□—□) 0.05 ml enzyme. Experimental conditions as in Fig. 1. Duration, 1 h.

ratio was different for different monoglycerides (see below). However, for any bile salt concentration up to 20 mM, it was possible to obtain extensive hydrolysis if sufficient amphiphile was present. From the present experiments, however, a direct effect of bile salts on the enzyme cannot be excluded.

A typical cationic detergent, cetyl-trimethyl ammonium bromide, considerably

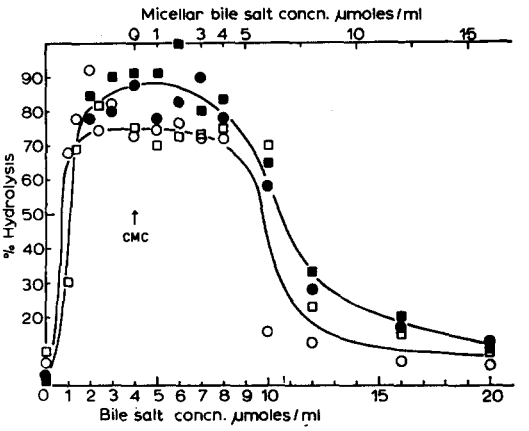

Fig. 6. Effect of sodium taurocholate (■—■, □—□), or sodium glycocholate (●—●, ○—○) on hydrolysis of micellar 1-monoolein, 6 μmoles/ml. Upper curve (●—●, ■—■) 0.2 ml lipase. Lower curve (○—○, □—□) 0.05 ml lipase. Experimental conditions as in Fig. 1. Duration, 1 h. The upper abscissa indicates the concentration of micellar bile salt. Extensive hydrolysis occurs below the CMC. The curves in Fig. 5 and Fig. 6 are superimposable if plotted with micellar bile salt concentration as abscissa.

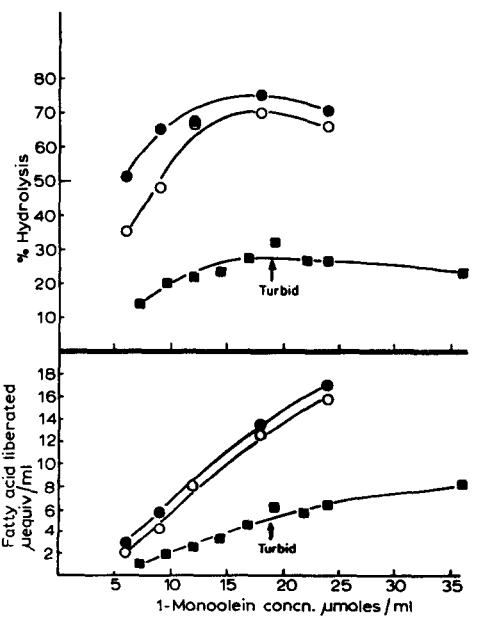

Fig. 7. Extent of hydrolysis, above, and fatty acid liberation, below, of micellar 1-monoolein with increasing A_m/D_m ratio. ■—■, 0.2 ml enzyme; ○—○, 0.4 ml enzyme; ●—●, 0.8 ml enzyme. Sodium glycodeoxycholate, 12 μmoles/ml. Duration of experiment, 1 h. Experimental conditions as in Fig. 1.

decreased the rate of hydrolysis of dispersed or micellar monoolein. About a tenfold increase in enzyme concentration was necessary to obtain similar rates of hydrolysis.

Effect of substrate concentration

From the preceding, it is apparent that substrate concentration cannot be considered in terms of the monoglyceride concentration alone. If the monoglyceride is increased while the bile salt concentration is held constant, the composition of the micelle changes; its charge per unit area decreases and it is possible there are significant changes in molecular arrangement[14]. The results of such experiments in which monoglyceride concentration was increased with constant bile salt concentration are shown in Fig. 7, for three different enzyme concentrations. With higher monoolein concentration (and higher A_m/D_m ratio) there is increased hydrolysis. When the system became turbid, the extent of hydrolysis seemed to remain roughly constant despite further increases in the A_m/D_m ratio.

A more reasonable way to increase the substrate concentrations is to increase the concentration of micelles of identical composition, experimentally done by increasing the monoglyceride and bile salt concentrations in such a way as to hold the A_m/D_m ratio constant. The experimental results are shown in Fig. 8. There was a decrease in extent of hydrolysis although the fatty acid liberation was roughly constant.

Experiments with different monoglycerides and structural analogues

1-Monoglycerides: It has been shown that C_{18} unsaturated 1-monoglycerides and the saturated 1-monoglycerides, C_{12}, C_{10}, and C_8, are solubilized in bile salt solution as amphiphiles with high saturation ratios[18]. The micelle is liquid[21] and there is general agreement that pancreatic lipase attacks only liquid substrates[1]. Therefore it might be predicted that in buffer alone pancreatic lipase would attack liquid substrates if they were sufficiently dispersed that the enzyme could reach the ester bond. Such

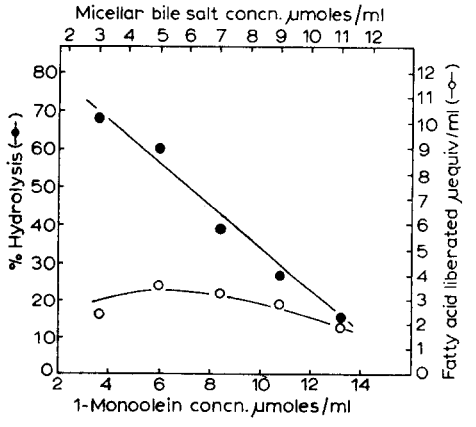

Fig. 8. Extent of hydrolysis (●—●), and fatty acid liberation (○—○), with increasing concentration of 1-monoolein and sodium glycodeoxycholate, the A_m/D_m ratio being held constant. Duration of experiment, 1 h. Conditions as in Fig. 1.

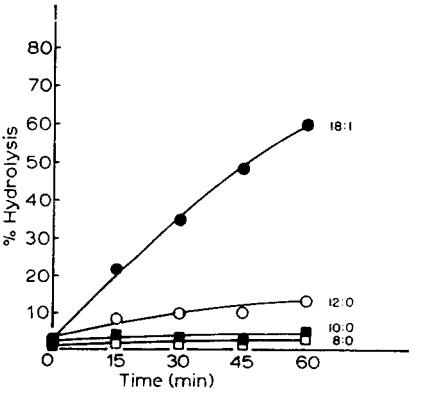

Fig. 9. Time course of hydrolysis of 1-monoolein (18:1), 1-monolaurin (12:0), 1-monodecanoin (10:0) and 1-monooctanoin (8:0). Sodium glycodeoxycholate and monoglycerides, each 6 μmoles/ml; experimental conditions as in Fig. 1.

was observed but only to some extent. 1-Monopalmitin, 1-monodecanoin and 1-monomyristin are dispersed poorly in solid form in buffer; they are not hydrolyzed. 1-Monolaurin is dispersed in an emulsified form in buffer; it shows a limited hydrolysis. 1-Monoolein is liquid crystalline in buffer. When it was dispersed by bile salt below its CMC, but not solubilized, it was extensively hydrolyzed (Fig. 6).

In bile salt solution, with the appropriate A_m/D_m ratio, 1-monoolein was readily attacked. 1-Monopalmitin and 1-monomyristin were hydrolyzed to a very limited extent, but these compounds have a very low solubility in the bile-salt micelle. The solubility of 1-monopalmitin in the bile-salt micelle can be greatly increased by the addition of 1-monoolein[20]. Under these conditions, 1-monopalmitin was readily cleaved. Experiments with mixed 1-monopalmitin-1-monoolein- or 1-monoolein-1-monolaurin micelles showed that oleic acid was preferentially liberated in both cases[22]. These experiments do not necessarily indicate that pancreatic lipase hydrolyzes micellar 1-monoolein more rapidly than micellar 1-monolaurin, for the availability of the ester linkages of fatty acids of different chain length or conformation may not be the same.

Middle chain 1-monoglycerides were not hydrolyzed at A_m/D_m ratios where 1-monoolein was readily split (Fig. 9). This effect was not explained by their higher solubility in buffer, and was therefore studied in more detail with 1-monodecanoin. Fig. 10 shows that 1-monodecanoin was hydrolyzed at very high A_m/D_m ratios.

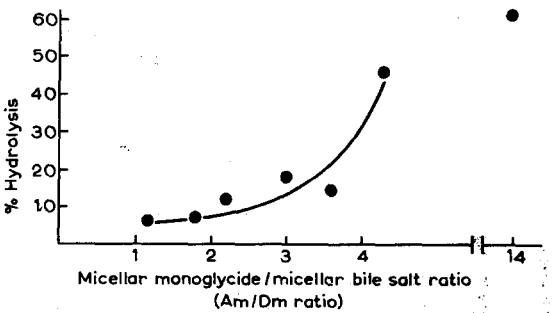

Fig. 10. Extent of hydrolysis of 1-monodecanoin with increasing A_m/D_m ratio. Sodium glycodeoxycholate, 3.6 μmoles/ml; lipase, 0.4 ml. Duration of experiment, 1 h. Experimental conditions as in Fig. 1.

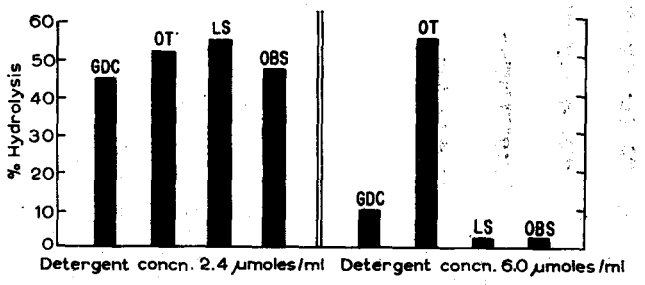

Fig. 11. Comparison of the effect of bile salts and different anionic detergents on the extent of hydrolysis of 1-monodecanoin at different A_m/D_m ratios. In all, 1-monodecanoin, 6 μmoles/ml. Left, bile salt or detergent concentration, 2.4 μmoles/ml. Right, 6 μmoles/ml. GDC, sodium glycodeoxycholate; OT, sodium oleyl taurate; LS, sodium lauryl sulfate; OBS, sodium *p*-(*n*-octylbenzene) sulfonate. Duration of experiment, 1 h. Experimental conditions as in Fig. 1.

1-Monolaurin was also hydrolyzed if the A_m/D_m ratio was increased. The necessary A_m/D_m ratio was higher than that necessary for 1-monoolein hydrolysis, but considerably below that necessary for 1-monodecanoin hydrolysis.

Bile salts and several anionic detergents behaved similarly at high A_m/D_m ratio (Fig. 11). At low ratios, *i.e.*, at higher detergent concentrations, hydrolysis was observed only with sodium oleyl laurate. This detergent is characterized by a high saturation ratio for the non-polar solute azobenzene[14] but a low saturation ratio for the polar solute monoolein. It may be postulated to form a very disordered micelle.

1-Monodecanoin was also hydrolyzed in the simple two-phase system heptane–buffer without added bile salt. In this system the physical state of all monoglycerides should be somewhat comparable and interestingly enough all monoglycerides showed similar rates of hydrolysis (Fig. 12). The problem of physical state is inherent in these experiments also. It cannot be assumed for the shorter chain, less amphipathic monoglycerides, that all of the monoglyceride present in the system was concentrated at the heptane–water interface.

1-α,α-Dimethylmonodecanoin was not hydrolyzed by pancreatic lipase in agreement with previous experiments in which ester bonds containing this branched chain fatty acid radical were not split by pancreatic lipase[23].

Structural analogues: Glycol monooleate was readily hydrolyzed. The extent of hydrolysis was similar to that of 1-monoolein. However, inhibition by bile salt was not observed until much lower A_m/D_m ratios.

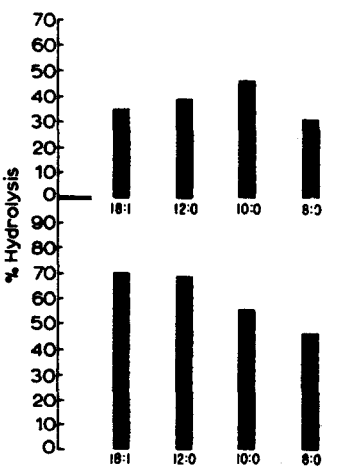

Fig. 12. Hydrolysis of 1-monoolein (18:1), 1-monolaurin (12:0), 1-monodecanoin (10:0) and 1-monooctanoin (8:0) at a heptane–buffer interface. Above: 6 μmoles monoglyceride, 0.25 ml heptane, 5.0 ml buffer. Below, 12 μmoles monoglyceride, 0.5 ml heptane, 5.0 ml buffer. The buffer and experimental conditions were otherwise as in Fig. 1; duration of experiment, 1 h. 0.2 ml lipase was used.

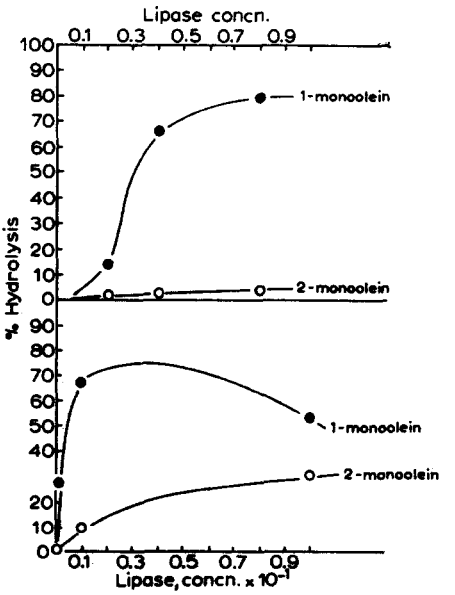

Fig. 13. Hydrolysis in 1 h of 2-monoolein (○—○) compared to that of 1-monoolein (●—●). Lower curve: 10 × enzyme concentration. In both, sodium glycodeoxycholate and monoglyceride, 6 μmoles/ml. Experimental conditions as in Fig. 1.

2-Monoglyceride: 2-Monoolein behaves as 1-monoolein in dilute bile-salt solution; its saturation ratio is identical[18]. Fig. 13 shows the hydrolysis of 2-monoolein using a favorable A_m/D_m ratio. The enzyme concentration has been varied over a hundred fold range. The 2-ester was not attacked at all, considering the 1-isomer present at the start of the incubation period as well as the isomerization occurring during the experiment[18], until very high enzyme concentrations. Thin-layer chromatography of the reaction products at the high enzyme concentrations where apparent hydrolysis took place showed the formation of di- and tri-glycerides. Thus, it is possible the hydrolysis occurring here represented cleavage of glyceride 1-ester linkages and not a direct attack at the 2-position. The results are in agreement with many indirect studies on the specificity of pancreatic lipase[24,25]

Effect of added fatty acids, alcohols or 1-mono-ethers: In all probability, the monoglyceride in the monoglyceride–bile salt micelle is oriented with its polar groups at the surface of the micelle. The fatty acid radical formed from hydrolysis of the monoglyceride probably does not reorient, although some change in the micellar size during hydrolysis is possible considering the smaller area occupied by a carboxyl group than esterified glycerol and the electrostatic repulsion of those carboxyl groups which ionize. These factors should oppose each other. Thus, during an experiment, the micellar surface is continuously changing. With time, it contains a higher percentage of carboxyl groups, some of which are ionized. It might therefore be predicted that the time course of hydrolysis would not be linear and that a decrease with time of the rate of hydrolysis would occur; this is observed in Fig. 1. It is assumed that all

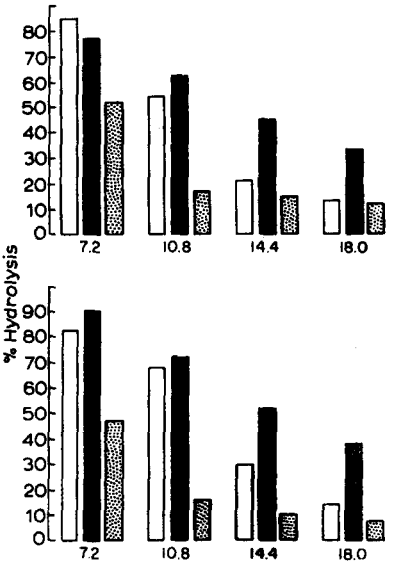

Fig. 14. Effect of added oleic acid (■—■) or glyceryl 1-monododecyl ether (□—□) on hydrolysis of micellar 1-monoolein. As a control, 1-monoolein (□—□) was added in identical concentrations. Upper diagram: 9 μmoles/ml total amphiphile. Concentration: 6 μmoles/ml; 1-monoolein plus 3 μmoles/ml added amphiphile. Lower diagram: 12 μmoles/ml total amphiphile. Concentration: 6 μmoles/ml 1-monoolein plus 6 μmoles/ml added amphiphile. Sodium glycodeoxycholate was used; its concentration in μmoles/ml is shown under each group of bars. Duration of experiment, 1 h. Experimental conditions as in Fig. 1.

micelles in the system are in complete and instantaneous equilibrium, *i.e.*, that the composition of all micelles changes simultaneously as hydrolysis progresses.

In Fig. 14 are shown the results of adding oleic acid to the 1-monoolein prior to incubation. As discussed, addition of oleic acid changes the A_m/D_m ratio, and controls of added 1-monoolein and added glyceryl 1-monododecyl ether were also run. The results showed an increased rate of hydrolysis when oleic acid was added prior to incubation; the increase was greater with oleic acid than with either of the other amphiphiles added as controls, and this effect was especially marked at higher bile-salt concentrations where hydrolysis is usually inhibited.

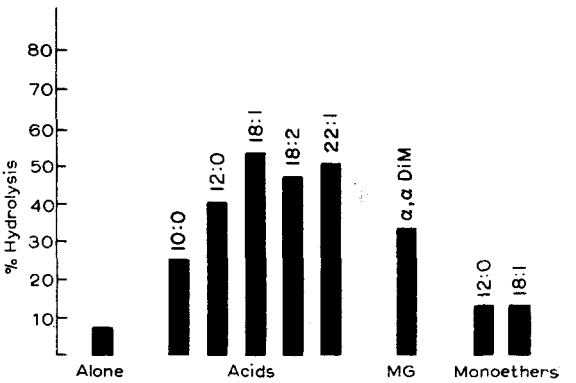

Fig. 15. Effect of added amphiphile on the hydrolysis of micellar 1-monodecanoin. Acids: decanoic (10:0), lauric (12:0), oleic (18:1), linoleic (18:2), erucic (22:1). Monoglycerides (MG): 1-α,α-dimethyl monodecanoin. Mono-ethers: glyceryl-1-monododecyl ether (12:0); glyceryl 1-monooleyl ether or selechyl alcohol (18:1). In all 6 μmoles sodium glycodeoxycholate/ml and 6 μmoles 1-monodecanoin/ml. 3 μmoles amphiphile added/ml. Duration of experiment, 1 h. Experimental conditions as in Fig. 1. As noted, the branched chain monoglyceride, 1-mono-α,α-dimethyl decanoin is completely resistant to hydrolysis by pancreatic lipase.

As noted previously, 1-mono-decanoin was not hydrolyzed at an A_m/D_m ratio where extensive hydrolysis of 1-monoolein occurred. The effect of added amphiphile on the rate of 1-monodecanoin hydrolysis at an otherwise unfavourable A_m/D_m ratio is seen in Fig. 15. An increase in the rate of hydrolysis occurred on addition of a variety of amphiphiles. Of interest is the increase caused by a variety of long-chain acids and the mono-ethers which can be considered as structural analogues of 1-monoglycerides. Addition of a branched chain monoglyceride, which itself is not hydrolyzed caused a marked increase in the hydrolysis rate.

DISCUSSION

It is clear that pancreatic lipase can attack substrates in a form other than an emulsion. We are not aware of any similar studies in which substrates have been used in well-characterized micellar solution, although the technique of dispersing (without further definition) of substrates in solutions of non-ionic detergents such as Tween is common. BANGHAM AND DAWSON[7–11] have presented detailed studies on lecithin hydrolysis, but their substrates were in the form of suspended myelin figures, or monolayers, rather than the isotropic solutions discussed here; indeed these workers

have presented evidence that enzymic activity occurred only at the interface of their insoluble substrates and not in the bulk phase[9].

Monoglycerides were used in this study, because of their striking solubility in bile salt solutions. Di- and triglycerides have a negligible solubility in bile salt solutions[20] and cannot be used. They do have a higher solubility in solutions of non-ionic detergents, and might be studied as micellar substrates in this fashion. The experimental approach is of practical value, as evidenced by the simple and direct demonstration of the specificity of pancreatic lipase for the 1-ester linkage. It was similarly useful for showing the susceptibility to hydrolysis of glycol 1-monooleate, as well as the resistance to hydrolysis of 1-α,α-dimethyl monodecanoin.

Whether hydrolysis by pancreatic lipase of micellar 1-monoglyceride occurs during fat digestion and absorption *in vitro* is unknown. However, it could only account for a small fraction of the hydrolysis of dietary fat considering the fact the monoglycerides found in intestinal content are chiefly of the 2-configuration[26].

The broad pH optimum 5–8 is in agreement with earlier work from this laboratory that the pH optimum of pancreatic lipase is lowered by the addition of bile salt[27]. The spontaneous hydrolysis of micellar 1-monoolein at pH 8.2 and above is of interest.

Although the addition of bile salts greatly increased the extent of hydrolysis, the experiments with the trihydroxy-conjugates showed clearly that this effect occurred below the CMC of the ternary system. The experiment suggests that the effect of bile salts might be chiefly attributed to their dispersing effect. The striking hydrolysis of the monoglyceride when present at the heptane–buffer interface indicates that another type of dispersion or orientation is possible. However, dispersion per se does not mean that extensive hydrolysis will occur; monolaurin is dispersed in buffer alone, yet hydrolyzed to a limited extent. Monooctanoin in molecular solution in buffer is not hydrolyzed. Thus for hydrolysis to occur, monoglycerides must not only be dispersed, but also in some type of association.

Cationic detergents as cetyl-trimethylammonium bromide disperse and solubilize monoglycerides but much higher concentrations of lipase are needed for the same rate of hydrolysis. These data indicate that pancreatic lipase can hydrolyze monoglyceride present in a strongly positive, as well as a strongly negatively charged micelle. As the micellar substrates described here are rather similar to the mixed monolayer substrates used by BANGHAM AND DAWSON in their studies of phospholipases, pancreatic lipase seems to be quite different from phospholipase B or C which only attack substrates having negatively or positively charged surfaces respectively[8,11].

When the inhibition of hydrolysis by high bile salt concentrations was observed, it was initially considered that the charge density on the micellar surface was the critical factor, *i.e.*, when the charge density exceeded a certain value, hydrolysis was inhibited (*cf.* ref. 7). This view, however, did not explain the limited hydrolysis of the middle-chain saturated 1-monoglycerides; nor did it explain why the critical A_m/D_m ratio for inhibition should be lower with sodium oleyl laurate than with other anionic detergents; nor did it explain the differences in the extent of hydrolysis of 1-monodecanoin caused by the addition of different amphiphiles (compare the branched chain monoglyceride with the mono-ethers in Fig. 15). A more satisfactory hypothesis is proposed; unfortunately, it is also more vague.

Evidence has been presented suggesting that the bile-salt micelle is more organized than ordinary detergent micelles[14]. The hypothesis is advanced that a certain

amount of disorder must be present in the micelle before the monoglyceride can be attacked by pancreatic lipase. Such disorder can be induced by (a) swelling the micelle with sufficient amphiphile, sufficient can only be defined circularly at the moment (b) adding another amphiphile which induces disorder; the marked effect of the branched chain monoglyceride in this respect is noteworthy or (c) using a detergent which forms more disordered micelles, the occurrence of hydrolysis with sodium oleyl laurate at A_m/D_m ratios where no hydrolysis occurs with bile salts or sodium *p*-(*n*-octylbenzene) sulfonate being an example.

The concept of order-disorder in the micelle is not a new one and some experimental evidence for it has been presented[28,29]. It can also be expressed as the tightness of packing of the polar groups of the molecules in the micelle. The packing arrangement for the bile-salt micelle cannot be discussed in any detail, as nothing is known of its molecular arrangement. Also, changes in packing concomitantly change the charge density to some extent.

Micellar substrates can be considered analogous to monolayer substrates, which have been used for the study of phospholipid hydrolysis[10], and it is of interest that the hydrolysis of phospholipid in a monolayer can be prevented by increasing the pressure of the monolayer, thus altering the packing of the polar groups. Lecithin is solubilized as an amphiphile in bile-salt micelles, and lecithin–bile-salt micelles might be a substrate for lecithinases.

The results of the titration experiments have physiological implications, as the monoglyceride, fatty acid, and bile salt composition and concentration of the micellar solution used were comparable to those present in human intestinal content during fat digestion and absorption[20,30]. The data indicate that some fraction of the fatty acid in intestinal content during fat digestion and absorption will be ionized, as small intestinal content usually has a pH between 6 and 7 (see ref. 30). The presence of soaps in intestinal content was also predicted by SCHMIDT-NIELSEN[31] who titrated fatty acids solubilized in alkyl sulfate solution. However, we have not found any previous report of such titrations of a fatty acid solubilized in a detergent micelle, other than that of SCHMIDT-NIELSEN, despite the simplicity of the experimental procedure.

The pK_a' of unsubstituted long-chain fatty acids, neglecting all solubility considerations, and assuming no molecular association, should be about 4.8 (see ref. 31). It has been known for many years that the dissociation constant of a fatty acid at an oil–water interface is several units higher[32]. VEIS AND HOERR[32] showed that the pK of a long-chain quarternary amine in water was lowered when micelle formation occurred. The well-known change in $\Delta pK_a'/\Delta$ concentration in solutions of anionic detergents when micellar aggregation occurs[34] is also evidence for an increase in the pK_a' of anionic detergents when they are present in the micellar state.

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REFERENCES

1 P. DESNUELLE, *Advan. Enzymol.*, 23 (1961) 129.
2 A. S. C. LAWRENCE, in K. DURHAM, *Surface Activity and Detergency*, Macmillan and Co., London, 1961, p. 158.
3 A. S. C. LAWRENCE, *Soap, Perfumery Cosmetics*, 33 (1960) 1177.
4 H. E. GARRET, in K. DURHAM, *Surface Activity and Detergency*, Macmillan and Co., London, 1961, p. 29.
5 G. S. HARTLEY, in R. T. HOLMAN, W. O. LUNDBERG AND T. MALKIN, *Progr. in the Chem. of Fats and Other Lipids*, Vol. 3, Pergamon Press, London, 1955, p. 19.
6 H. B. KLEVENS, *Chem. Revs.*, 47 (1950) 1.
7 A. D. BANGHAM AND R. M. C. DAWSON, *Nature*, 182 (1958) 1292.
8 A. D. BANGHAM AND R. M. C. DAWSON, *Biochem. J.*, 72 (1959) 486.
9 R. M. C. DAWSON AND A. D. BANGHAM, *Biochem. J.*, 72 (1959) 493.
10 A. D. BANGHAM AND R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 133.
11 A. D. BANGHAM AND R. M. C. DAWSON, *Biochim. Biophys. Acta*, 59 (1962) 103.
12 G. S. HARTLEY, Aqueous Solutions of Paraffin-chain Salts, *Actualités Sci. Indust. 387*, Hermann et Cie., Paris, 1936, p. 1.
13 A. F. HOFMANN, *Nature*, 190 (1106) 1961.
14 A. F. HOFMANN, *Biochem. J.*, 17 (1963) 173.
15 A. NORMAN, *Arkiv Kemi*, 8 (1955) 331.
16 A. F. HOFMANN, *Acta Chem. Scand.*, in the press.
17 B. BORGSTRÖM, *Biochemical Problems of Lipids*, Butterworths Scientific Publ. London, 1955, p. 179.
18 A. F. HOFMANN, *Biochim. Biophys. Acta*, 70 (1963) 305.
19 H. K. MANGOLD, *J. Am. Oil Chemists Soc.*, 38 (1961) 708.
20 A. F. HOFMANN AND B. BORGSTRÖM, *Federation Proc.*, 21 (1962) 43.
21 K. SHINODA AND E. HUTCHINSON, *J. Phys. Chem.*, 66 (1962) 43.
22 A. F. HOFMAN, B. BORGSTRÖM AND G. ARVIDSON, unpublished experiments.
23 S. BERGSTRÖM, B. BORGSTRÖM, N. TRYDING AND G. WESTÖÖ, *Biochem. J.*, 58 (1954) 604.
24 F. H. MATTSON, J. H. BENEDICT, J. B. MARTIN AND L. W. BECK, *J. Nutr.*, 48 (1952) 335.
25 B. BORGSTRÖM, *Biochim. Biophys. Acta*, 13 (1954) 491.
26 A. F. HOFMANN, *J. Lipid Res.*, 3 (1962) 391.
27 B. BORGSTRÖM, *Biochim. Biophys. Acta*, 13 (1954) 149.
28 H. B. KLEVENS, *J. Chim. Phys.*, 49 (1952) 615.
29 J. H. SCHULMAN, W. STOECKENIUS AND L. M. PRINCE, *J. Phys. Chem.*, 63 (1959) 1677.
30 B. BORGSTRÖM, A. DAHLQVIST, G. LUNDH AND J. SJÖVALL, *J. Clin. Invest.*, 36 (1957) 1521.
31 K. SCHMIDT-NIELSEN, *Acta Physiol. Scand.*, 12 (1946) sppl. 37.
32 R. A. PETERS AND R. W. WAKELIN, *Trans. Faraday Soc.*, 34 (1938) 1537.
33 A. VEIS AND C. W. HOERR, *J. Colloid Sci.*, 15 (1960) 427.
34 A. S. C. LAWRENCE AND M. P. MCDONALD, in J. SCHULMAN, *Proc. Second Intern. Congr. Surface Activity*, Butterworths Sci, Publications, London, 1957, p. 385.

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THE EXTRACTION AND PURIFICATION OF LIPOGENIN

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SUMMARY

A method for the extraction and purification of lipogenin has been described. By means of alcohol fractionation, chromatography, electrophoresis, and mannanase treatment an approx. 1000-fold purification was achieved. The purified material contained some 30 % carbohydrate, mainly mannan, and 70 % of a peptide.