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Non-enzymic protein induced hydrolysis of *p*-nitrophenyl acyl esters in relation to lipase/esterase assays

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Protein-induced hydrolysis of *p*-nitrophenyl acyl esters has been studied mainly via the reaction between bovine serum albumin (BSA) and *p*-nitrophenyl myristate. In the present study BSA caused a large increase in the amount of *p*-nitrophenol released (measured as increase in absorbance at 410 nm) at pH above 6.5, but at lower pH BSA had only negligible catalytic activity. BSA-catalysed hydrolysis of *p*-nitrophenyl acyl esters increased with temperature (25–80°C), concentration of BSA and concentration of *p*-nitrophenyl acetate, but was virtually unaffected by changes in *p*-nitrophenyl myristate concentration. The rate of hydrolysis was highest for *p*-nitrophenyl acyl esters with an acyl moiety of 8–12 carbon atoms at a *p*-nitrophenyl concentration (0.028 mmol/litre) below critical micelle concentration (CMC) for all *p*-nitrophenyl acyl esters tested. For a *p*-nitrophenyl acyl ester concentration (0.28 mmol/litre) above CMC (acyl moiety of ≥6 carbon atoms) the rate of hydrolysis decreased with increasing chain length of the acyl moiety. The catalytic effect of BSA decreased with heat treatment (90–95°C for 15 min) of the protein solution, but did not disappear. Tween-20 effectively retarded BSA-catalysed hydrolysis of *p*-nitrophenyl myristate, and the effect was dependent on both Tween-20 and BSA concentrations. Free fatty acids displayed a similar but less pronounced inhibitory effect on the hydrolytic reaction. The inhibitory effect increased with the length of the fatty acids (C-4 to C-10).

INTRODUCTION

Lipase activity is important in many food products and has received increasing interest within the last decade. The accelerated hydrolysis of the triglycerides present leads to both desirable and undesirable changes. Lipase activity may contribute directly and indirectly to flavour and flavour development in cheeses, fermented sausages and dry-cured hams (Berdagué *et al.*, 1993; El-Soda & Pandian, 1991; Molina et al., 1991; Næs *et al.*, 1993) but can also be responsible for spoilage of oat kernels and milk (Ekstrand *et al.*, 1993; Law, 1979).

In general, the activity of lipases/esterases in foods is low, so sensitive methods are necessary to detect their activity. Sensitive methods employ either conventional titration assays consisting of different kinds of triglycerides in emulsion, or assays containing artificial substrates composed of a chromophore or a fluorophore linked to a fatty acid through an ester linkage. The latter are normally preferred as they are time-saving and easy to perform. Thus, artificial substrates have been used increasingly in the examination of lipase/esterase activity in many foods and in evaluation of lipase/esterase activity of starter cultures for meat and cheese products. Investigation of lipase/esterase activity using artificial substrates in such systems often includes samples with a high protein content; e.g. screening experiments (Hinrichsen *et al.*, 1994), first steps in purification procedures (Lee & Lee, 1989) and evaluation of lipase/esterase activity in fresh pork meat and dry-cured ham (Motilva *et al.*, 1992) etc. However, non-enzymic proteins have been found to catalyse the hydrolysis of artificial substrates under similar conditions (Brandl & Zizer, 1973; Casida & Augustinsson, 1959; Downey & Andrews, 1965; Koga *et al.*, 1982; Kokubo *et al.*, 1982; Rongone *et al.*, 1956; Tove, 1962; Wolfbeis & Gürakar, 1987). Hydrolytic action of non-enzymic proteins in lipase/esterase assays using artificial substrates may therefore contribute to the hydrolysis of these substrates and thereby overestimate the lipase/esterase activity in samples tested. If artificial substrates, such as chromophore or fluorophore derivatives of fatty acids, are used in assays to estimate lipase/esterase activity in these food-related systems, it is necessary to account for this non-enzymic effect. This can best be achieved by understanding the mechanism behind the hydrolytic action of non-enzymic proteins.

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The present study investigates the effect of non-enzymic hydrolysis of *p*-nitrophenyl acyl esters in relation to measurements of lipase/esterase activity in high protein containing samples. Long chain fatty acid esters of *p*-nitrophenol were chosen for the following reasons: (i) they are less soluble in aqueous solutions and form emulsion-like solutions which favour measurement of lipase activity as opposed to esterase activity, and (ii) the reactivity of long chain fatty acid esters can be compared with the reactivity of *p*-nitrophenyl acetate (Brandl & Zizer, 1973; Casida & Augustinsson, 1959; Downey & Andrews, 1965) and *p*-nitrophenyl caproate (Koga *et al.*, 1982) from previous investigations. The difference in reactivity between these different *p*-nitrophenyl acyl esters may help in elucidating the mechanism of non-enzymic hydrolysis of *p*-nitrophenyl acyl esters.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA, fraction V, 99% pure, protease free), β-lactoglobulin, γ-globulin, metmyoglobin, Tween-20, butyric acid, *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl caproate, *p*-nitrophenyl caprylate, *p*-nitrophenyl caprate, *p*-nitrophenyl laurate, *p*-nitrophenyl myristate and *p*-nitrophenyl palmitate were obtained from Sigma. Dimethyl sulfoxide (DMSO), caproic acid, caprylic acid and capric acid were purchased from Fluka. Whey protein was obtained from Grindsted Products (Denmark). Analytical grade chemicals and double deionised water were used throughout.

Assay conditions

p-Nitrophenyl acyl esters were dissolved in DMSO (30 mmol/litre, except for *p*-nitrophenyl acetate and *p*-nitrophenyl myristate (27–108 mmol/litre)) and diluted 1:100 with 20 mmol/litre buffer (glycin/HCl, acetate, Tris/phosphate, phosphate, Tris or glycin/NaOH). This solution/emulsion is referred to as the *assay solution.* Reactions were initiated by mixing 100 ml BSA solutions (1–30 mg/ml in 20 mmol/litre of the respective buffer or double deionised water in the experiment regarding influence of pH) with 1.1 ml assay solution, resulting in a final volume of 1.2 ml. Unless stated otherwise, assay conditions consisted of: 20 μmol/litre Tris buffer (pH 8.0); 0.28 mmol/litre *p*-nitrophenyl myristate and 25.2 μmol/litre BSA which were incubated 30 min in a waterbath (50°C). Blanks were performed with 100 μl buffer instead of BSA solution. *p*-Nitrophenyl acyl ester hydrolysis was measured by estimating the release of *p*-nitrophenol by increase in absorbance at 410 nm using a Shimadzu UV-2101PC spectrophotometer equipped with a temperature-controlled cuvette compartment. All experiments were performed in duplicate. Results are given in concentration of released *p*-nitrophenol (mmol/litre) using a standard curve or in percentage of maximal reaction rate in the specific experiment. Data were corrected for the effect of pH on the extinction coefficient of *p*-nitrophenol and for spontaneous hydrolysis under the specified conditions.

Protein catalysed hydrolysis of *p*-nitrophenyl myristate

Assays were performed using protein concentrations of 1.67 mg/ml with the exception of metmyoglobin (0.167 mg/ml). Blanks for whey protein and metmyoglobin were prepared by adding 100 μl of either solution to 1.1 ml incubated assay solution to compensate for these proteins' distinct absorption at 410 nm.

BSA-catalysed hydrolysis of *p*-nitrophenyl myristate

Assays were performed using BSA concentrations of 12.6 and 25.2 μmol/litre (with or without heat treatment for 15 min, at 90–95°C). Reactions were followed for 30 min in a temperature-controlled cuvette (50°C).

pH profile

Assays were performed in 20 mmol/litre glycin/HCl buffer, acetate buffer, Tris/phosphate buffer or glycin/NaOH buffer (pH 4–10), using a BSA concentration of 12.6 μmol/litre. After incubation the assay solutions were diluted with 1.2 ml 1 mol/litre glycin/NaOH-buffer (pH 9.0) prior to measuring their absorbance.

Temperature profile

Assays were performed in 20 mmol/litre phosphate buffer (pH 7.0 and 8.0) with a BSA concentration of 25.2 μmol/litre. Samples were incubated 30 min (pH 7.0: 40–80°C; pH 8.0: 25–45°C).

Effect of BSA concentration

Assays were performed with BSA concentrations of 0–25.2 μmol/litre using *p*-nitrophenyl acetate or *p*-nitrophenyl myristate as substrate. Samples containing *p*-nitrophenyl acetate were incubated 10 min (35°C) and samples containing *p*-nitrophenyl myristate were incubated 30 min (50°C).

Effect of *p*-nitrophenyl acyl ester concentration

The effect of *p*-nitrophenyl acyl ester concentration was tested using *p*-nitrophenyl acetate or *p*-nitrophenyl myristate. Assays were performed using a BSA concentration of 6.3 μmol/litre and *p*-nitrophenyl acyl esters at concentrations of 0–1.0 mmol/litre. Samples containing *p*-nitrophenyl acetate were incubated 10 min (35°C) and samples containing *p*-nitrophenyl myristate were incubated 30 min (50°C).

Effect of chain length in *p*-nitrophenyl acyl esters

Assays were performed using BSA concentration of 6.3 μmol/litre and *p*-nitrophenyl acyl esters: *p*-nitrophenyl

acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl caproate, *p*-nitrophenyl caprylate, *p*-nitrophenyl caprate, *p*-nitrophenyl laurate, *p*-nitrophenyl myristate or *p*-nitrophenyl palmitate at concentrations of 0.028 mmol/litre or 0.28 mmol/litre. Samples were incubated 30 min (35°C). *p*-Nitrophenyl acyl esters with a fatty acid chain length longer than 16 C-atoms were not used due to their low solubility in aqueous media.

Effect of Tween-20

Assays were performed using BSA concentrations of 0–37.8 μmol/litre and Tween-20 at concentrations of 0–1%.

Effect of free fatty acids or Tween-20 on BSA-induced hydrolysis of *p*-nitrophenyl myristate

Assays were performed using a BSA concentration of 25.2 μmol/litre. Tween-20 or free fatty acids (butyric acid, caproic acid, caprylic acid or capric acid) were added to the assay solution at concentrations of 3–30 mmol/litre or 30 mmol/litre, respectively. Solutions containing fatty acids were adjusted with 1 mol/litre NaOH to pH 8.0.

RESULTS

Protein-catalysed hydrolysis of *p*-nitrophenyl myristate

All the proteins tested showed a catalytic effect on the hydrolysis of *p*-nitrophenyl myristate with BSA being the most effective and γ-globulin the least effective catalyst (Table 1).

BSA-catalysed hydrolysis of *p*-nitrophenyl myristate

Figure 1 shows the release of *p*-nitrophenol during incubation of 12.6 and 25.2 μmol/litre BSA with *p*-nitrophenyl myristate for 30 min (50°C). The rate of *p*-nitrophenol release decreased during the incubation. Heat treated BSA (15 min, 90–95°C) showed reduced catalytic activity, but higher activity than the control.

Table 1. *p*-Nitrophenol released during incubation of different proteins with *p*-nitrophenyl myristate. Reaction conditions: 0.167 mg/ml metmyoglobin or 1.67 mg/ml BSA, β-lactoglobulin, γ-globulin or whey protein; 0.28 mmol/litre *p*-nitrophenyl myristate; 20 mmol/litre Tris buffer, pH 8.0; 30 min incubation at 50°C

Protein	*p*-Nitrophenol released (mmol/litre) after 30 min incubation
BSA	0.099 (100)[b]
γ-globulin	0.006 (6)
β-lactoglobulin	0.033 (33)
Whey protein	0.018 (18)
Metmyoglobin	0.039[a] (39)

[a]Value multiplied by 10 to match the other protein concentrations. [b]Values in brackets give the released *p*-nitrophenol in percentage of released *p*-nitrophenol for BSA.

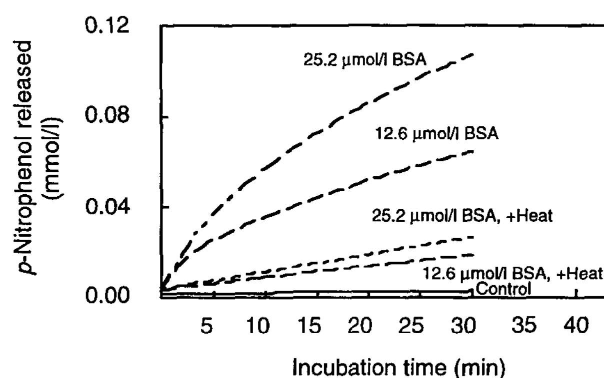

Fig. 1. *p*-Nitrophenol released during incubation of heated and unheated BSA with *p*-nitrophenyl myristate. Reaction conditions: 0.0 (control), 12.6, 25.2 μmol/litre BSA, with and without heat treatment (15 min at 90–95°C); 0.28 mmol/litre *p*-nitrophenyl myristate; 20 mmol/litre Tris buffer, pH 8.0; 30 min incubation at 50°C.

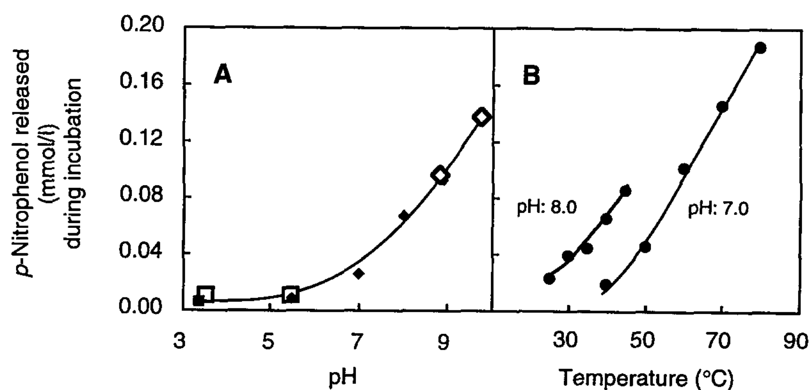

Fig. 2. Effect of pH and temperature on release of *p*-nitrophenol in the reaction between BSA and *p*-nitrophenyl myristate. Reaction conditions: (A) 12.6 μmol/litre BSA; 0.28 mmol/litre *p*-nitrophenyl myristate; 20 mmol/litre; (■) Glycin/HCl buffer; (□) Acetate buffer; (◆) Tris/phosphate butter; (◇) Glycin/NaOH buffer; 30 min incubation at 50°C. (B) 25.2 μmol/litre BSA; 0.28 mmol/litre *p*-nitrophenyl myristate; 20 mmol/litre phosphate buffer pH 7.0 or pH 8.0; 30 min incubation. The data in panel B are well described by the Arrhenius equation. Activation parameters, calcuated according to the transition state theory are $\Delta H^{\neq}_{298K}$: 53.5 and 51.6 kJ/mol for pH 7.0 and 8.0, respectively.

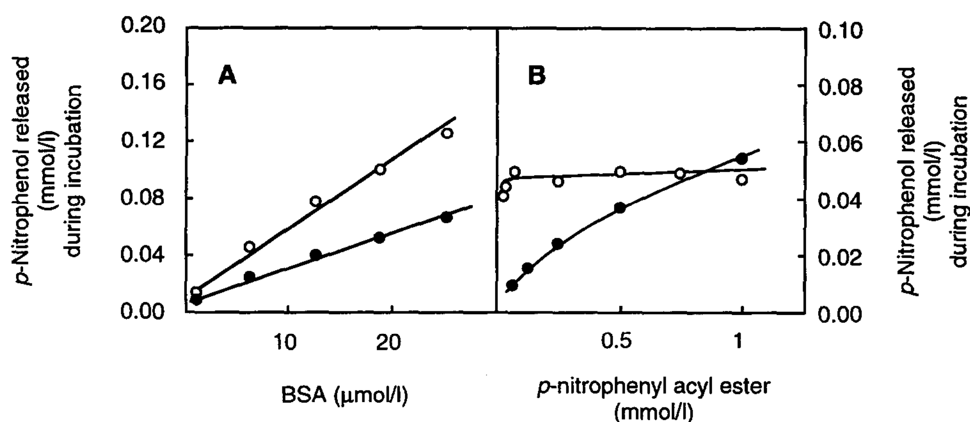

Fig. 3. Effect of concentration of *p*-nitrophenyl acetate (●) or *p*-nitrophenyl myristate (○) and concentration of BSA on release of *p*-nitrophenol. Reaction conditions: A: 0–25.2 µmol/litre BSA; 0.28 mmol/litre *p*-nitrophenyl acetate or *p*-nitrophenyl myristate; 20 mmol/litre Tris buffer, pH 8.0; 10 min incubation at 35°C (*p*-nitrophenyl acetate); 30 min incubation at 50°C (*p*-nitrophenyl myristate). (B) 6.3 µmol/litre BSA; 0–1.0 mmol/litre *p*-nitrophenyl acetate or *p*-nitrophenyl myristate; 20 mmol/litre Tris buffer, pH 8.0; 10 min incubation at 35°C (*p*-nitrophenyl acetate); 30 min incubation at 50°C (*p*-nitrophenyl myristate).

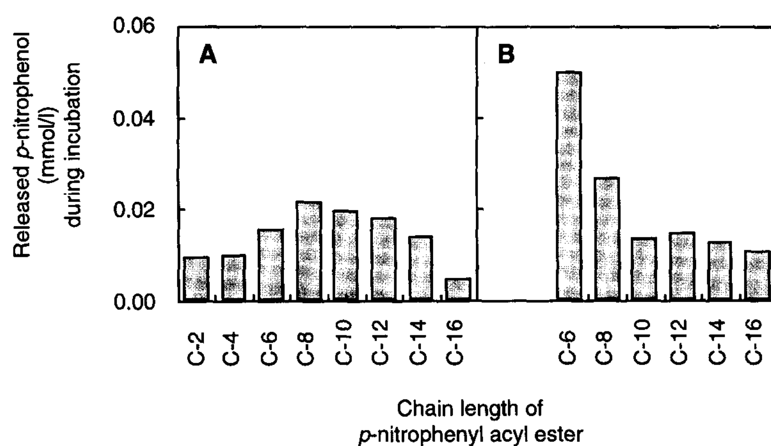

Fig. 4. *p*-Nitrophenol released during incubation of BSA and *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl caproate, *p*-nitrophenyl caprylate, *p*-nitrophenyl caprate, *p*-nitrophenyl laurate, *p*-nitrophenyl myristate or *p*-nitrophenyl palmitate. Reaction conditions: (A) 6.3 µmol/litre BSA; 0.028 mmol/litre *p*-nitrophenyl acylester; 20 mmol/litre Tris buffer, pH 8.0; 30 min incubation at 35°C. (B) Identical to (A) but with a *p*-nitrophenyl acyl ester concentration of 0.28 mmol/litre.

Effect of temperature, pH and concentrations on the catalytic action of BSA on the hydrolysis of *p*-nitrophenyl acyl esters

Figures 2 and 3 show that BSA has a marked effect on the hydrolysis of *p*-nitrophenyl acyl ester and that the degree of reaction is affected by temperature, pH, concentration of BSA and concentration of *p*-nitrophenyl acetate.

The activity of BSA increased with temperature (Fig. 2B). BSA had negligible catalytic activity below pH 6.5, but caused a large increase in the amount of *p*-nitrophenol released above pH 6.5 (Fig. 2A).

Figure 3(A) shows the linear dependence between the hydrolysis of *p*-nitrophenyl acetate or *p*-nitrophenyl myristate and BSA concentration (0–25.2 mmol/litre). Hydrolysis of *p*-nitrophenyl acetate showed a similar but not linear dependence on the concentration of *p*-nitrophenyl acetate (0–20 mmol/litre), while hydrolysis of *p*-nitrophenyl myristate was virtually unaffected by the concentration of *p*-nitrophenyl myristate (0.1–1.0 mmol/litre) (Fig. 3B).

Effect of chain length in *p*-nitrophenyl acyl ester on BSA-induced hydrolysis

The effect of chain length of the acyl moiety in the *p*-nitrophenyl acyl ester on BSA-induced hydrolysis is shown in Fig. 4. At a *p*-nitrophenyl acyl ester concentration (0.028 mmol/litre) below critical micelle concentration (CMC) for all *p*-nitrophenyl acyl esters tested BSA-induced hydrolysis of *p*-nitrophenyl acyl esters was most effective with an acyl moiety of 8–12 carbon atoms. For a *p*-nitrophenyl acyl ester concentration (0.28 mmol/litre) above CMC (acyl moiety of ≥6 carbon atoms) the rate of hydrolysis decreased with increasing chain length of the acyl moiety.

Effect of free fatty acids or Tween-20 on BSA-induced hydrolysis of *p*-nitrophenyl myristate

Tween-20 effectively inhibited BSA catalysed hydrolysis of *p*-nitrophenyl myristate (Fig. 5). The degree of inhibition was dependent on both BSA and Tween-20 concentration, as an increase in BSA concentration needed

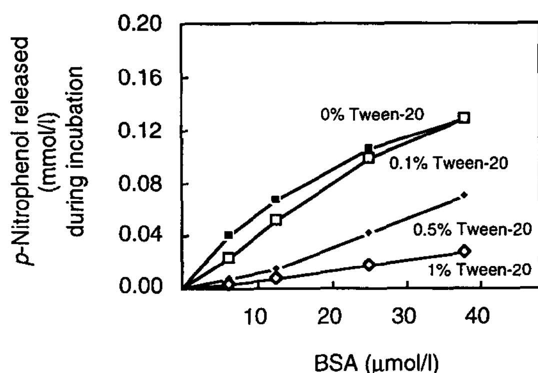

Fig. 5. Effect of Tween-20 on release of *p*-nitrophenol in the reaction between BSA and *p*-nitrophenyl myristate. Reaction conditions: 0–37.8 μmol/litre BSA; 0.28 mmol/litre *p*-nitrophenyl myristate; 20 mmol/litre Tris buffer, pH 8.0 with 0–1% Tween-20; 30 min incubation at 50°C.

a corresponding increase in Tween-20 concentration to obtain a similar level of inhibition.

Free fatty acids also displayed an inhibitory effect on BSA-catalysed hydrolysis of *p*-nitrophenyl myristate (Fig. 6). The inhibitory effect of free fatty acids increased with fatty acid chain length, however, the inhibitory effect of Tween-20 was much stronger than any of the fatty acids tested.

DISCUSSION

At a *p*-nitrophenyl acyl ester concentration of 0.28 mmol/litre, the appearance of the assay solutions depended on the *p*-nitrophenyl acyl ester used. Solutions containing *p*-nitrophenyl acyl esters with acyl moieties of 6 or more carbon atoms resulted in turbid solutions, while the shorter chain acyl esters gave transparent solutions. This indicates that assay solutions containing medium and long chain fatty acid esters of *p*-nitrophenol should be considered as mixtures of *p*-nitrophenyl acyl ester micelles and dissolved *p*-nitrophenyl acyl esters, while short chain fatty acid esters are true solutions. The latter solutions can be used to measure esterase activity while the former can mainly be used to measure lipase activity because they contain the substrate/water interfaces needed for the action of true lipases. This was further confirmed by the fact that a commercial lipase showed hydrolytic activity towards *p*-nitrophenyl myristate, but hardly any hydrolytic activity towards *p*-nitrophenyl acetate, while a commercial proteinase showed some esterase activity towards *p*-nitrophenyl acetate, but no activity towards *p*-nitrophenyl myristate (data not shown).

Data given in Table 1 and Figs 2 and 3 show that the activity of non-enzymic proteins will affect results obtained in enzyme assays for neutral and alkaline lipases/esterases that employ *p*-nitrophenyl acyl esters as substrates. Furthermore, heat treatment of BSA reduces its ability to induce hydrolysis of *p*-nitrophenyl acyl esters (Fig. 1). This requires caution if heat treatment of crude enzyme solutions is used to distinguish 'true' enzyme activity from any activity performed by other components.

The dependence of BSA-induced hydrolytic reactions on chain length was influenced by the concentration of *p*-nitrophenyl acyl esters. This was ascribed to the solubility of the substrate. *p*-Nitrophenyl acyl esters with a fatty acid chain length of at least six carbon atoms gave cloudy solutions at concentrations of 0.28 mmol/litre, but not at 0.028 mmol/litre. The BSA molecule probably only has catalytic activity towards soluble *p*-nitrophenyl acyl esters and not towards the fraction of *p*-nitrophenyl acyl esters which are incorporated in micelles. This is further verified by the observation that the rate of hydrolysis of *p*-nitrophenyl myristate, which forms micelles, was highly independent of the *p*-nitrophenyl myristate concentration, while the hydrolysis of *p*-nitrophenyl acetate, which is completely soluble at the concentrations tested, was dependent on the *p*-nitrophenyl acetate concentration. The dependence on chain length for the low concentration of *p*-nitrophenyl acyl esters gives information about hydrolytic activity of BSA against soluble *p*-nitrophenyl acyl esters as differences in solubility of the *p*-nitrophenyl acyl esters can be ignored under the used conditions. The results from the high concentration of *p*-nitrophenyl acyl esters will, however, give information about the action of non-enzymic proteins in enzyme assays, where 'high' substrate concentrations are normally used.

The inhibitory effect of free fatty acids on BSA-catalysed hydrolysis of *p*-nitrophenyl acyl esters may explain

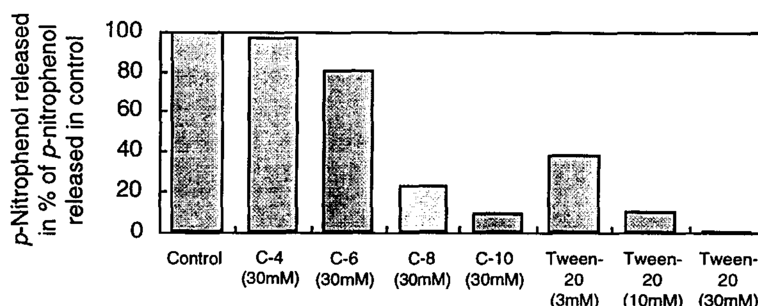

Fig. 6. Inhibitory effect of free fatty acids and Tween-20 on the reaction between BSA and *p*-nitrophenyl myristate. All data are given in percentage of the control (sample without addition of fatty acids or Tween-20). Reaction conditions: 25.2 μmol/litre BSA; 0.28 mmol/litre *p*-nitrophenyl myristate; 30 mmol/litre butyric acid, caproic acid, caprylic acid or capric acid; 3–30 mmol/litre Tween-20; 20 mmol/litre Tris buffer, pH 8.0; 30 min incubation at 50°C.

the decreased rate of *p*-nitrophenol liberation observed during incubation of BSA with *p*-nitrophenyl myristate (Fig. 1), as released myristic acid will subsequently cause product inhibition.

The observed inhibition of BSA catalysed hydrolysis of *p*-nitrophenyl myristate by Tween-20 might be used to overcome non-enzymic protein catalysed hydrolysis. However, experiments with a commercial lipase showed that Tween-20 could not be used to remove the non-enzymic protein (BSA) effect (data not shown), as no enzyme activity was detected under these conditions. This might be due to the fact that the amount of detergents added disturb the two-phase system in the assay, or that Tween-20 inhibits activity of the enzyme tested. However, detergents may still be able to prevent or reduce non-enzymic protein activity in enzyme assays using *p*-nitrophenyl acyl esters as substrates.

On the basis of the present data we suggest a mechanism for the hydrolytic action of BSA on *p*-nitrophenyl acyl esters. *p*-Nitrophenyl acyl esters are initially adsorbed onto the BSA molecule, making the ester bond more susceptible to hydrolysis. This increased susceptibility could be caused by, for example, interactions between the ester bond and different side chains on the protein, by increased nucleophilicity of water due to a hydrophobic area near the adsorption site, or by a water molecule held in a fixed position near the ester bond. The mechanism can explain why heat treatment diminishes the catalytic activity of BSA and why the reaction is dependent on the acyl moiety of the *p*-nitrophenyl acyl ester.

Heat treatment will result in conformational changes of the BSA molecule, altering the surface of the protein or decreasing the number of available adsorption sites. Both these factors will make it more difficult for *p*-nitrophenyl acyl esters to adsorb onto the BSA molecule.

The amount of *p*-nitrophenol released during BSA-induced hydrolysis was influenced by the acyl moiety in the *p*-nitrophenyl acyl ester. This, in combination with the observed effect of Tween-20 and the longer chain fatty acids tested, supports the view that it is the fatty acid moiety of *p*-nitrophenyl acyl esters which adsorbs to the BSA molecule. BSA had most effect on hydrolysis of *p*-nitrophenyl acyl esters with an acyl moiety of 8 to 12 carbon atoms, with the maximum hydrolytic activity measured for *p*-nitrophenyl caprylate. These findings are comparable with those of Tove (1962) and Wolfbeis & Gürakar (1987) who found that BSA-induced hydrolysis was highest for β-naphthyl caprylate and coumarin palmitate. The decrease in hydrolytic activity of BSA towards *p*-nitrophenyl myristate and *p*-nitrophenyl palmitate (Fig. 4A) is probably caused by steric hindrance of the acyl moiety.

This mechanism is equivalent to that proposed for the catalytic effect of BSA on the reaction between sorbic acid and mercaptoethanol (Wedzicha & Zeb, 1991; Wedzicha & Picard, 1993). In this mechanism sorbic acid is adsorbed onto the BSA molecule followed by a nucleophilic attack from mercaptoethanol. The authors noticed an inhibitory effect of the free fatty acid, hex-3-enoic acid, on the BSA catalysed reaction between sorbic acid and mercaptoethanol. This corresponds with our data and the data of Wolfbeis & Gürakar (1987), who found an inhibitory effect of lauric acid on the BSA catalysed hydrolysis of coumarin acyl esters.

CONCLUSION

Non-enzymic proteins must be expected to contribute to the overall enzyme activity of neutral or alkaline lipases/esterases in assays that use *p*-nitrophenyl acyl esters as substrates if the samples have a high protein content. Contribution from non-enzymic proteins can not be compensated for by traditional heat inactivation of the enzyme because non-enzymic protein activity is also affected by heat treatment. Introduction of an appropriate detergent might be the only way to minimise or eliminate non-enzymic protein activity in *p*-nitrophenyl acyl ester based lipase/esterase assays.

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