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A thermoactive uropygial esterase from chicken: Purification, characterisation and synthesis of flavour esters

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ABSTRACT

A lipolytic activity was located in the chicken uropygial glands, from which a carboxylesterase (CUE) was purified. Pure CUE has an apparent molecular mass of 50 kDa. The purified esterase displayed its maximal activity (200 U/mg) on short-chain triacylglycerols (tributyrin) at a temperature of 50 °C. No significant lipolytic activity was found when medium chain (trioctanoin) or long chain (olive oil) triacylglycerols were used as substrates. The enzyme retained 75% of its maximal activity when incubated during 2 h at 50 °C. The NH_2-terminal amino acid sequence showed similarities with the esterase purified recently from turkey pharyngeal tissue. Esterase activity remains stable after its incubation during 30 min in presence of organic solvents such as hexane or butanol. CUE is a serine enzyme since it was inactivated by phenylmethanesulphonyl fluoride (PMSF), a serine-specific inhibitor. The purified enzyme, which tolerates the presence of some organic solvent and a high temperature, can be used in non-aqueous synthesis reactions. Hence, the uropygial esterase immobilised onto $CaCO_3$ was tested to produce the isoamyl and the butyl acetate (flavour esters). Reactions were performed at 50 °C in presence of hexane. High synthesis yields of 91 and 67.8% were obtained for isoamyl and butyl acetate, respectively.

1. Introduction

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are the enzymes catalysing the hydrolysis of ester bonds and are widely distributed in animals, plants and microorganisms [1,2]. In organic media, they catalyse reactions such as esterification, inter-esterification and trans-esterification [3]. Esterases and lipases were first differentiated on the basis of their substrate specificity. It was established that esterase activity was restricted to aqueous solutions of short acyl chain esters whereas lipases specially hydrolysed emulsions of water insoluble triacylglycerols [4]. Most of these lipases and esterases can be used in organic solvents in organic synthesis and in various industrial applications (detergent industry, oleochemical industry, pulp and paper industry and resolution of chiral drugs) [5–8].

Lipolytic enzymes including esterases and lipases have a highly conserved catalytic triad, which is mostly composed of Ser, Asp/Glu, and His residues. In addition, they have a pentapeptide sequence motif, GXSXG, around the active site serine. The enzymes display the common α/β hydrolase fold [9], which is commonly found in other hydrolases, such as serine carboxypeptidase [10].

The uropygial gland is one of the integumentary glands present in birds. It is a bilobate sebaceous organ, variable both in shape and size [11] and located at the base of the bird's tail feathers. This gland produces a large amount of volatile and nonvolatile substances in a waxy fluid that is spread on feathers as a part of plumage maintenance [12]. These oily materials are believed to play a role in feather wear or water proof plumage.

Recently, Cherif et al. [13] have purified an avian esterase from turkey (*Meleagris gallopavo*) pharyngeal tissue. However, to our knowledge, no lipolytic enzymes from bird uropygial glands have been characterised so far. The present work describes the purification of an esterase from the uropygial gland of domestic chicken. We studied the catalytic properties and the stability of the uropygial esterase and we evaluated its potential use in the synthesis of two industrially important flavour esters, isoamyl acetate and butyl acetate.

2. Material and methods

2.1. Materials

Tributyrin (TC_4, 99%; puriss), benzamidine and phenylmethanesulfonyle fluoride (PMSF) were from Fluka (Buchs, Switzerland).

Abbreviations: CUE, chicken uropygial esterase; CPL, chicken pancreatic lipase; PMSF, phenylmethanesulphonyl fluoride; $CaCO_3$, calcium carbonate; FPLC, Fast protein liquid chromatography.

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Tripropionin (TC_3, 99%, GC) was from Janssen Chimica (Geel, Belgium). Vinyl propionate and vinyl acetate were from Aldrich (Steinheim, Allemagne). Trioctanoin (TC_8), Triacetin (TC_2), bovine serum albumine (BSA), sodium deoxycholate (NaDC) and nitrocellulose membrane were from Sigma Chemical (St. Louis, USA). Gum Arabic was from Mayaud Baker LTD (Dagenham, UK). Acylamide and bis-acrylamide electrophoresis grade were from BDH (Poole, UK). Marker proteins and supports of chromatography used for CUE purification: Sephacryl S-200 and Mono-Q sepharose were from Pharmacia (Uppsala, Sweden) and Bio-sil SEC-125 (HPLC) filtration column (250 mm × 4.6 mm) were purchased from Knauer (Germany). PVDF membrane and protein sequencer Procise 492/610A provided from Applied Biosystems (Roissy, France). pH-Stat was from Metrom (Herisau, Switzerland).

2.2. Lipases

Chicken pancreatic lipase (CPL) was purified to homogeneity at our laboratory as previously described [14].

2.3. Uropygial gland collection

Chicken uropygial glands were collected immediately after death from the local slaughterhouse of Sfax. A stock of chicken uropygial gland was kept at −20 °C. The organs were dissected. Homogenisation was performed for (2 × 30 s) using a Waring blender. After centrifugation, lipolytic activity was measured in the clear supernatant of the homogenated gland.

2.4. Determination of lipolytic activity

The lipolytic activity was measured titrimetrically at pH 8.5 and 50 °C with a pH-stat, under the standard assay conditions described previously, using tributyrin (TC_4) assay: 0.25 ml tributyrin in 30 ml of 2.5 mM Tris–HCl, pH 8.5. Lipase activity was also measured using triacetin (TC_2) (0.25 ml), tripiopionin (TC_3) (0.25 ml) or trioctanoin (TC_8) in 30 ml of 2.5 mM phosphate buffer pH 7.3 according to Ferrato et al. [4]. The enzymatic hydrolysis of solutions and emulsions of vinyl esters was followed potentiometrically at 25 °C and pH 7. Assays were performed in 30 ml of 2.5 mM Tris–HCl buffer pH 7.0 containing 0.1 M NaCl. Standard conditions for measuring enzyme activity at increasing concentrations of esters have been described previously [2]. Lipolytic activity was expressed as international units. One unit corresponds to 1 μmol fatty acid released/min. Specific activities are expressed as units/mg of protein.

2.5. Determination of protein concentration

Protein concentration was determined as described by Bradford [15] using BSA ($E^{1\%}_{1cm} = 6.7$) as reference.

2.6. Esterase purification

2.6.1. Tissue extraction

After defrosting, chicken uropygial glands deprived of the most of their gross fat were cut in 1 cm pieces. CUE extract was prepared by soaking 40 g of uropygial tissue in buffer A (25 mM Tris–HCl, 150 mM NaCl, pH 8, 1 mM Benzamidine) at 4 °C for 30 min. After centrifugation for 30 min at 8500 rpm, the supernatant was used as starting material for subsequent purification steps.

2.6.2. Ammonium sulphate precipitation

The supernatant containing 1450 UT was brought to 60% saturation with solid ammonium sulphate under stirring conditions and maintained at 4 °C during 30 min. After centrifugation (10,000 rpm during 20 min at 4 °C), the precipitate was resuspended in 15 ml of buffer A. Insoluble material was removed by centrifugation at 10,000 rpm during 10 min.

2.6.3. Heat treatment

The supernatant was incubated during 5 min at 50 °C. After rapid cooling, insoluble denatured proteins were removed by centrifugation during 20 min at 12,000 rpm. The recovery of CUE activity from the supernatant was of about 80%.

2.6.4. Filtration on Sephacryl S-200

The supernatant containing the esterase activity was loaded on a column (2.5 × 150 cm) of gel filtration Sephacryl S-200 equilibrated with buffer B (25 mM Tris–HCl, 25 mM NaCl, pH 8, 1 mM Benzamidine). Elution of enzyme was performed with the same buffer at a rate of 36 ml/h. The fractions containing the esterase activity (eluted between 1.1 and 1.2 a void volume) were pooled (Fig. 1A).

2.6.5. FPLC anion exchange chromatography

The pooled fractions of Sephacryl S-200 were concentrated and injected to the FPLC column Mono-Q gel (trimethylammonium anion exchange) equilibrated in buffer B. The column (1 cm × 10 cm) was rinsed with 40 ml of the same buffer. No lipolytic activity was detected in the washing flow. Adsorbed proteins were eluted with a linear NaCl gradient (160 ml of 50–450 mM in buffer B) at a rate of 2 ml/min.

2.7. Analytical methods

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulphate was performed by the method of Laemmli [16]. Samples for sequencing were electroblotted according to Bergman and Jörnvall [17]. Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature.

2.8. NH_2-terminal sequence analysis

To determine the NH_2-terminal sequence, protein bands from SDS gels were transferred to a problott membrane (Applied Biosystems). Automated Edman protein degradation was performed with a protein sequencer (Applied Biosystems Protein sequencer Procise 492) [18].

2.9. Effect of temperature on esterase activity and stability

The optimum temperature for the CUE activity was determined by carrying out the enzyme assay at different temperatures (37–60 °C) at pH 8.5. The thermal stability was studied by incubating the esterase at various temperatures (37–70 °C) for 1 h and measuring, after centrifugation, the residual activity under standard titrimetric assay condition, using tributyrin as substrate.

2.10. Effects of organic solvents

In order to determine the effects of various organic solvents on esterase activity, the purified enzyme was incubated during 30 min in presence of an organic solvent. Then the residual activity was measured under standard conditions.

2.11. Effect of PMSF on esterase activity

The effect of phenylmethanesulphonyl fluoride, a serine-specific inhibitor, on esterase activity was tested by incubating the enzyme at room temperature (25 °C) for 1 h in the presence of 1 and 5 mM of PMSF. The residual enzyme activity was measured under the standard assay conditions.