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PURIFICATION OF PHOSPHOLIPASE A_2 FROM PORCINE SKELETAL MUSCLE

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Introduction

An intense lipolysis was observed during the processing of dry-cured ham. There was a close relationship between the decrease of phospholipids and the accumulation of free fatty acid (Buscaihon, Gandemer & Monin, 1994; Motilva, Toldra, Nieto & Flores, 1994). These observations indicate that phospholipases A plays an important role in lipolysis of dry-cured hams. The main activities are related to basic phospholipases A (Alasnier & Gandemer, 2000). Phospholipase A_2 has been mainly studied on various snake and bee venoms, and also mammalian pancreas and heart, because phospholipase A_2 plays a central role in diverse cellular processes including phospholipids digestion and metabolism, host defense and signal transduction (Kathryn et al., 1998). However, data on phospholipases A in skeletal muscle is limited. Only two studies were devoted to phospholipase A activity in mammalian skeletal muscles, in which phospholipase A activities were compared in the extracted solutions from different metabolic types of muscles (Hernandez, Navarro & Toldra, 1998; Alasnier & Gandemer, 2000). Up to now, pure phospholipase A has not yet been obtained from porcine skeletal muscles.

Objectives

The objective of this study was to purify phospholipase A_2 from porcine skeletal muscle for better understanding the nature of phospholipases A and for further understanding the lipolysis mechanism of the dry-cured ham.

Methodology

Preparation of muscle supernatant

Biceps femoris muscle from pig was homogenized (3×10 s spells at 20,000 rpm with ice cooling) in 4 vols of 0.1M Tris-HCl buffer, pH 8.5, containing 0.1mM PMSF. The homogenate was stirred for 30 min at 4 ºC and centrifuged for 20 min at 10,000×g. The supernatant was the source of the crude phospholipase.

Purification of the enzyme

The supernatant (10 ml) was applied to a 2.6×20 cm DEAE-Sephacel column equilibrated previously with 50 mM Tris-HCl buffer, pH 8.5 (buffer I). After washing with 200 ml buffer at a flow rate of 1.5 ml/min, the column was eluted with the same buffer containing 1M NaCl. The fractions containing enzyme activity were collected, concentrated and dialyzed for 12h against 20 mM Tris-HCl buffer, pH 8.0 (buffer II). The dialyzed enzyme was applied to a Blue -sepharose 6 F.F. column (1.0×8 cm) equilibrated previously with buffer II. After washing with the same buffer at a flow rate of 0.5 ml/min, the column was eluted with the same buffer containing 1M NaCl. The fractions with enzyme activity were pooled, concentrated and dialyzed against 20 mM sodium phosphate buffer (pH 6.8) (buffer III). The resulting sample was applied onto a Heparin-sepharose CL-6B column (1.0×8 cm) equilibrated previously with buffer III. The unadsorbed material was washed out with 20 ml buffer III at a flow rate of 0.5 ml/min, and the adsorbed material was eluted with 20 ml buffer III containing 150 mM NaCl, then, eluted with 1 M NaCl and 3 mg/ml heparin in buffer III.

Assay of phospholipase A2 activity

A radiochemical assay was used to monitor phospholiase A_2 activity in fractions from chromatography (Lumb et al., 1990; Alasnier et al., 2000). The substrate L-α-1-palmitoyl-2-[1-^{14}C] arachidonyl sn-glycerophatidylcholine (Perkin Elmer Life Sciences, specific activity 1.89 GBq mmol^{-1}) together with unlabelled L-α-1-palmitoyl-2-arachidonyl sn-glycerophatidylcholine (Sigma Chemical Co.) was solubilised in toluene:ethanol (1:1, v/v). The concentration of substrate was adjusted to 250 µmol/l phosphatidylcholine and 300 MBq l^{-1}. For the majority of studies 200 µl enzyme was incubated with 5 µl substrate at 40 ℃ in a shaking water bath for 20 min. The reaction was terminated by the addition of 2 ml fatty acid extraction mixture (toluene: chloroform: methanol 1.0: 0.5: 1.2 v: v: v), followed by 40 µl of 1.0 mol/l NaOH to ensure alkaline conditions. After vigorous vortexing the samples were centrifuged (1000×g for 10 min) to form the aqueous and organic phases. 200 µl upper aqueous phase was extracted to a scintillation vial containing 4 ml liquid scintillation cocktail (optiphase 'supermix', Perkin Elmer Life Sciences), radioactivity was counted in a liquid scintillator (LS 6000 IC, Beckman). Each sample was measured in triplicate. All results were corrected for the blank. Phospholipase A_2 activity was expressed as nmol fatty acids released per hour per mg protein (nmol h^{-1} mg^{-1} protein).

Results & Discussion

On a DEAE-Sephacel column, two active peaks were eluted from the adsorbed and unadsorbed fractions, respectively (Fig.1). These observations suggest that there are at least two types of phospholipase A_2 in porcine skeletal muscle. The second active protein peak was pooled and further purified on Blue-sepharose 6 F.F. and Heparin-sepharose CL-6 B affinity chromatography. The elution profiles are shown in Fig 2 and 3. The fractions with phospholipase A_2 activity on Heparin-sepharose CL-6 B affinity chromatography were pooled and an aliquot subjected to SDS-PAGE. A single protein band was observed (Fig. 4) and the molecular mass of the enzyme was estimated to be 66

kDa under conditions with β-mercaptoethanol. The results of purification of phospholipase A_2 from porcine skeletal muscle are summarized in Table 1. The enzyme was finally purified about 226-fold with 7.6% of recovery.

The purification of phospholipase A_2 in the first active peak of DEAE- Sephacel column and the characterization of properties of the purified enzyme are ongoing.

Conclusions

A phospholipase A_2 from porcine skeletal muscle was purified to electrophoretic homogeneity.

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Tables and Figures

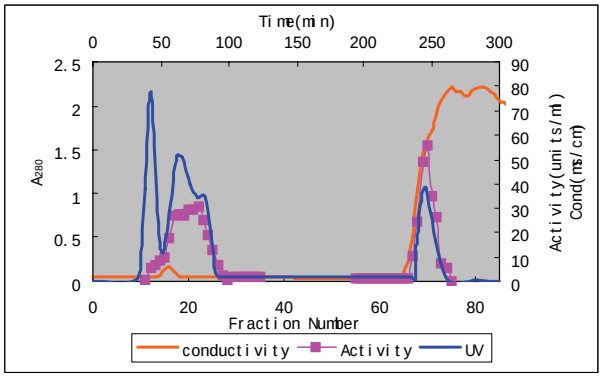

Fig.1 DEAE-Sephacel chromatography of the muscle supernatant

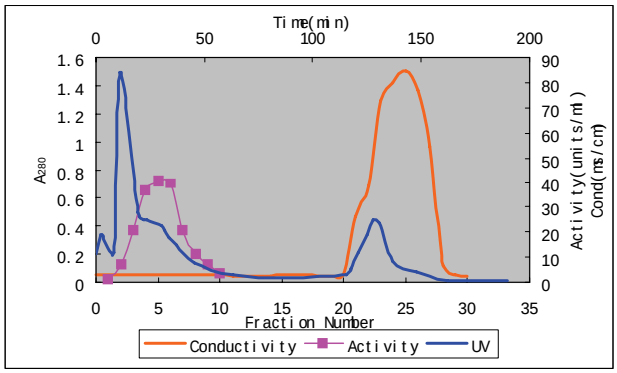

Fig.2 Blue-sepharose 6 F. F. chromatography of the active fractions obtained from DEAE-Sepharose chromatography (the second active peak in Fig.1)

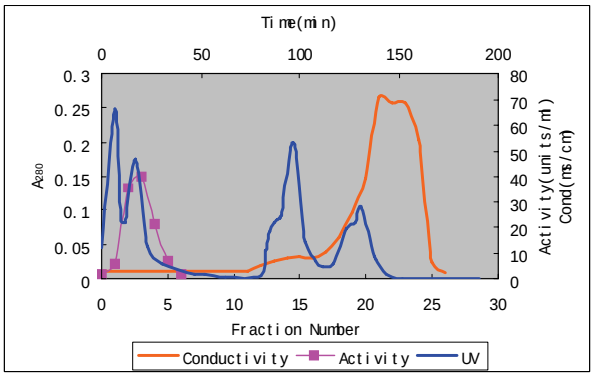

Fig.3 Heparin-sepharose CL-6 B chromatography of the active fractions obtained from Blue-sepharose 6 F. F. chromatography

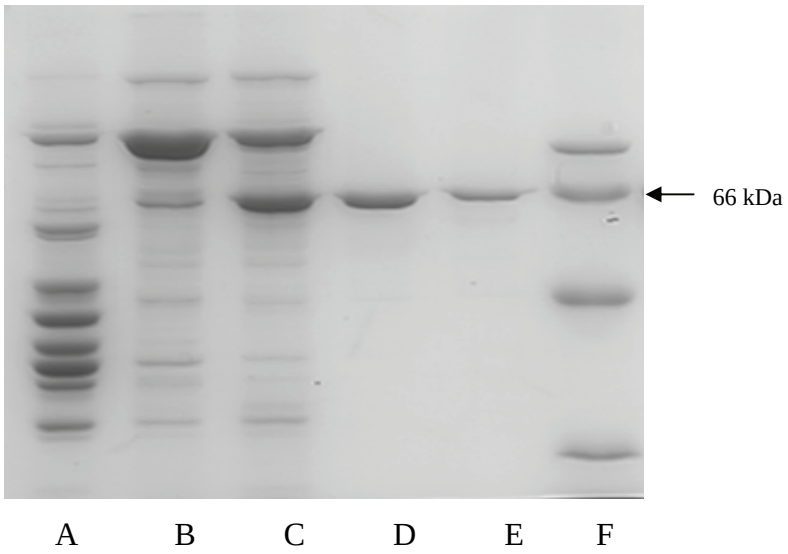

Fig.4 Profiles of SDS PAGE: (A) muscle supernatant; (B) the active peak 2 on DEAE-Sephacel chromatography; (C) the active fraction of Blue-sepharose 6 F.F. chromatography; (D-E) the active fraction of Heparin-sepharose CL-6 B chromatography, the purified phospholipase A_2 ; (F) low MW standard.
The concentration of acrylamide was 12%.

Table 1. Summary of the purification of phospholipase A_2 from porcine skeletal muscle

procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	3550	29110	8.2	1	100
DEAE-Sephacel	410	16605	40.5	4.94	57.0
Blue-sepharose 6 F.F	30	10836	361.2	44.05	37.2
Heparin-sepharose CL-6 B	1.2	2223.84	1853.2	226.00	7.64

One unit of enzyme activity was defined as the amount of enzyme releasing 1nmol fatty acids per hour per mg protein.