IMMUNOLOGICAL EVIDENCE FOR THE IDENTITY OF THREE PROTEINASES FROM RAT SKELETAL MUSCLE

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Received 16 December 1981

1. Introduction

The enzymatic capacity of proteinases from rat skeletal muscle with optimum activity in the alkaline pH range increases during several hormone-mediated protein catabolic conditions like starvation [1,2], insulin deficiency [1–3], testosterone deficiency [4], glucocorticoid treatment [1] as well as during muscle dystrophy [4,6,7]. With the obvious correlation between enhancement of enzyme activity and muscle protein breakdown, we assume that this proteinase system is involved in pathological muscle wasting.

Alkaline proteinases from rat skeletal muscle have been isolated, purified and characterized by several groups. The 'muscle alkaline protease' (MAP) isolated from the myofibrillar fraction has been characterized in [8]. The properties of this chymotrypsin-like enzyme are very similar to those of the alkaline proteinase [9] designated 'group specific proteinase' (GSP), and shown to immunologically crossreact with the mast cell chymase I [10].

In our laboratory, a chymotrypsin-like proteinase called 'cytoplasmic proteinase' (CP) has been isolated from the post-myofibrillar fraction of rat skeletal muscle, a proteolytic activity shown to be similar to both of the above enzymes [11,12].

Here, immunological methods were used to establish whether the 3 chymotrypsin-like enzymes, MAP, GSP and CP, are identical.

2. Experimental

For the isolation of enzyme, pooled hindleg skeletal muscles from male Wistar rats ~200 g body wt were used. The 'muscle alkaline proteinase' was isolated as in [8,13]. The 'group-specific protease' was purified as in [14] but omitting the last crystallization step. The 'cytosolic proteinase' was prepared as in [11]. The purity of all 3 enzyme preparations was assessed by SDS–polyacrylamide gel electrophoresis [15]. Analysis of gels revealed a single protein band with each purified proteinase.

Antisera to CP were raised in rabbits by intradermal injection of 0.1 mg enzyme protein suspended in complete Freund's adjuvants followed by an intravenous booster injection (0.1 mg enzyme) after 4 weeks. One week later the animals were bled. The IgG fraction was isolated from the antiserum by DEAE-Affigel blue chromatography (BioRad Labs., München) on a 2.5 × 20 cm column by the procedure described by the manufacturer. The isolated IgG fraction was further purified by gel filtration on a 2 × 100 cm column of Ultrogel AcA 34 (LKB, Bromma) equilibrated with PBS-buffer (0.15 M NaCl/0.01 M sodium phosphate (pH 7.4)). Using this procedure complete separation of the serum proteinase inhibitory activity, the serum proteolytic activity and the IgG fraction was achieved.

Immuno-inhibition tests were performed by incubation of 0.5 ml of proteinase solution with 0.5 ml anti-CP–IgG solution. Buffers used to dilute the antiserum were 1.1 M kJ/6 mM $Na_2S_2O_2$ (pH 8.0), 0.25 M K-phosphate (pH 8.0) and 1 M KCl/50 mM Tris–HCl (pH 8.5) when testing MAP, GSP and CP, respectively. After a 60 min incubation at 37°C, the antigen–antibody mixture was centrifuged for 10 min at 45 000 × *g*. Residual proteolytic activity was determined by incubation of 0.2 ml supernatant fraction with 0.05 ml 3% azocasein solution for 60 min at 37°C as in [16]. Proteolytic activity is given as % of the control containing proteinase solution and the corresponding buffer solution.

Published by Elsevier Biomedical Press
0014593/82/0000–0000/$02.75

Double immunodiffusion was performed in 1% agarose gels in PBS-buffer. After 3 days diffusion precipitin lines were stained with 0.2% Coomassie brilliant blue R-250 dissolved in methanol–acetic acid–H_2O (9:2:9, v/v/v) and destained in the same solution but without the dye.

3. Results

Incubation of the 3 alkaline proteinases CP, MAP and GSP with increasing amounts of the IgG fraction of the anti-CP antiserum resulted in a 90–100% inhibition of the enzymatic activity of all 3 enzymes. 50% inhibition of the enzymes was achieved with 50–70 μg IgG/U proteinase (fig.1).

Double immunodiffusion tests showed that CP was precipitated by the antiserum forming a single precipitin line. Single precipitin lines were also formed when the anti-CP antiserum was tested by double immunodiffusion against MAP and GSP, respectively. These precipitin lines completely fused with that formed between CP and anti-CP IgG (fig.2).

4. Discussion

Immunological techniques have often been used to study structural relationship of proteolytic enzymes [17]. Here, double immunodiffusion as well as immuno-inhibition experiments have shown complete immunological identity of the 3 chymotrypsin-like proteinases MAP, GSP and CP, isolated from rat skeletal muscle tissue by 3 different procedures as described by 3 different groups.

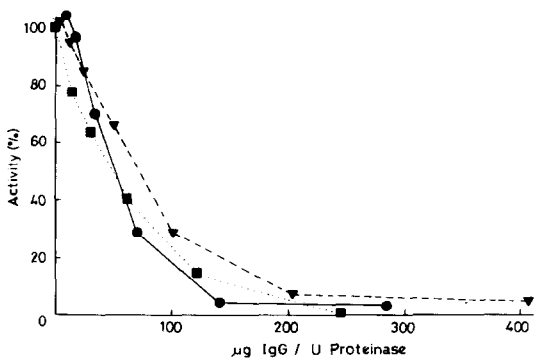

Fig.1. Inhibition of the enzymatic activity of the purified proteinases CP (●——●), GSP (▼——▼) and MAP (■——■) with anti-CP–IgG. Residual proteolytic activity was tested with azocasein as substrate.

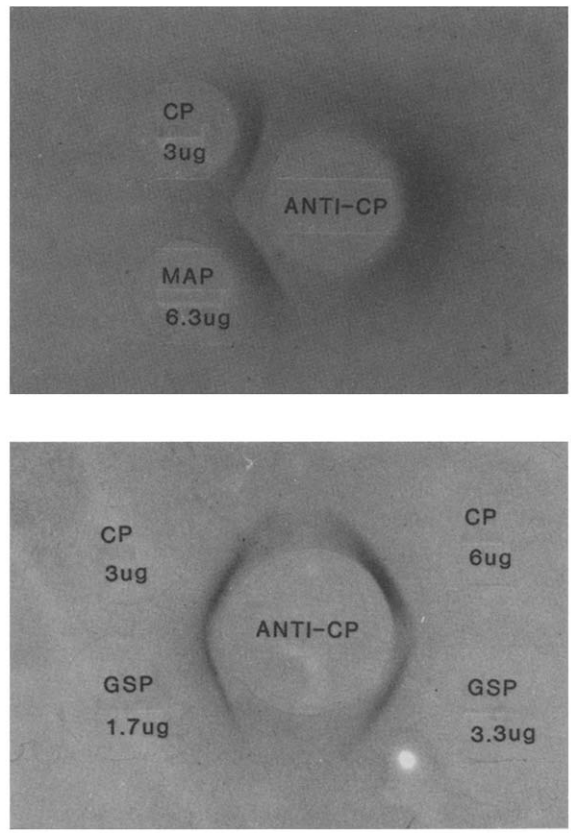

Fig.2. Double immunodiffusion of anti-CP–IgG (1.6 mg) against purified CP, MAP and GSP.

In addition to this immunological identity the 3 enzymes have been shown to share a number of properties; e.g., a high ionic strength requirement for their solubility, $M_r \sim 25\,000$, a chymotrypsin-like splitting specificity and their ability to be affected by proteinase inhibitors like di-isopropylfluorophosphate, soybean- and limabean-trypsin-inhibitor, tosyl-phenylalaninechloromethylketone and chymostatin [8,9,11,13].

Recent immunohistochemical experiments have shown that anti-CP–IgG is reacting with material appertaining to mast cells distributed in the muscle tissue rather than with muscle cell components [18]. In [10] GSP was shown identical with mast cell chymase I; in [13] similar properties of MAP and mast cell chymase were reported; thus, the complete immunological identity of MAP, GSP and CP and their largely

identical enzymatic properties suggest identity of the 3 enzymes and their mast cell origin.

Experiments are presently performed to investigate the function of this alkaline proteinase, particularly its role in hormone-induced pathological muscle wasting conditions.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 113), by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, Düsseldorf and by the Ministerium für Jugend, Familie und Gesundheit, Bonn.

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