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Identification of the goldfish 20S proteasome β6 subunit bound to nuclear matrix

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Abstract Proteasomes are large, multisubunit particles that act as the proteolytic machinery for most of the regulated intracellular protein breakdown in eukaryotic cells. Proteasomes are present in both the nucleus and cytoplasm. When we analyzed the molecular composition of protein constituents of the nuclear matrix preparation of goldfish oocytes by two-dimensional polyacrylamide gel electrophoresis followed by sequence analysis, we found a 26 kDa spot identical in amino acid sequence to the β6 subunits of the 20S proteasome. No spot of other subunits of 20S proteasome was detected. Here we describe the cloning, sequencing and expression analysis of *Carassius auratus*, *β6_ca*, which encodes one of the proteasome β subunits from goldfish ovary. From the screening of an ovarian cDNA library, two types of cDNA were obtained, one 941 bp and the other 884 bp long. The deduced amino acid sequences comprise 239 and 238 residues, respectively. These deduced amino acid sequences are highly homologous to those of β6 subunits of other vertebrates. Immunoblot analysis of nuclear matrix using anti-proteasome antibodies showed only a spot of *β6_ca*. These results suggest that the β6 subunit of the goldfish 20S proteasome, *β6_ca*, is responsible for anchoring proteasomes in the nucleus.

Key words: β6 subunit; Proteasome; Nuclear matrix

1. Introduction

Eukaryotic cells, from yeast to human, contain non-lysosomal large proteases called proteasomes [1]. The proteasomes are part of the ubiquitin-dependent proteolytic system, which regulates proteins through a mechanism of selective degradation [2–5]. The 26S proteasome is a functional complex. The core proteinase of this complex is the 20S proteasome, which comprises four stacked rings composed of two outer α- and two inner β-type subunit groups. Each group has seven different subunits [6]. The proteasomes are thought to be involved in the regulation of the meiotic and mitotic cell cycle [7]. Cell cycle dependent changes in the localization of proteasomes have been demonstrated. The translocation of proteasomes between cytoplasm and nucleus is thought to be important for functions of proteasomes [8–12].

The oocyte nucleus (germinal vesicle, GV) of immature oocytes is extremely large, an advantage for its isolation, and can be used for biochemical analysis. When we analyzed the molecular composition of protein constituents of the nuclear matrix prepared by high-salt and detergent extraction from GV of goldfish oocytes by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by amino acid sequence analysis, we found a 26 kDa spot identical in sequence to the C5 subunit of 20S proteasome. No spot corresponding to other 20S proteasome subunits whose molecular mass range from 23 to 31 kDa was observed. Then we conducted molecular cloning and immunological analysis of the C5 subunit of goldfish. This is the first report to identify a proteasomal subunit which may be involved in anchoring proteasomes in the nucleus.

2. Materials and methods

2.1. cDNA cloning of β6_ca

A polymerase chain reaction (PCR) fragment was amplified from goldfish ovary cDNA with primers *β6_ca* #1 (CARGAYWSYCCHAARTGYTA) and *β6_ca* #2 (TCYTTDGTVACRATRCARAT) (R = A or G; Y = C or T; W = A or T; S = G or C; H = A, C or T; D = A, G or T; V = A, C or G). The amplified PCR fragment was subcloned into pBluescript II SK(−) vector and sequenced. The PCR fragment was amplified using digoxigenin (DIG) DNA labeling mixture (Boehringer) to use as a probe. A λZAPII goldfish ovarian cDNA library was screened using the DIG-labeled probe. A total of 2×10^5 plaques were blotted onto the Hybond N^+ nylon membranes (Amersham) and hybridized at 60°C in hybridization solution (5×SSC, 0.5% blocking reagent (Boehringer), 0.1% *N*-lauroyl sarcosine, 0.02% SDS) with the DIG-labeled probe. After two rounds of hybridization, positive plaques were isolated. Plasmid DNA was prepared by the in vivo excision protocol using the ExAssist/SOLR system (Stratagene). DNA sequencing was performed using a 377A DNA sequencer (Perkin Elmer ABI) with the Dye Terminator Cycle Sequencing Kit (Perkin Elmer ABI).

2.2. RT-PCR (reverse transcription-polymerase chain reaction)

Total RNA from various kinds of goldfish tissues was isolated using Isogen (Wako) according to the manufacturer's instructions. Two μg of the total RNA was used for reverse transcription by the RT-PCR kit (Ready To Go, Amersham Pharmacia). The amplification of fragments of *β6_ca*-A was performed with a pair of primers; P1, 5′-CAGTGGAGCACAAGTTCTCT-3′ (nucleotides 156 to 175), and P2, 5′-TTGTTTTCTCCAGTTGCAT-3′ (nucleotides 844 to 862). That of *β6_ca*-B was performed with the primers P1 (nucleotides 147 to 166) and P3, 5′-CCAGCCCGTTTCCCTTCAG-3′ (nucleotides 813 to 831). The thermal cycle profile was 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, for 25 cycles.

2.3. Expression of β6_ca in bacteria and production of polyclonal antibody

The full-length ORF of *β6_ca* was amplified by PCR with primers designed to produce a *Nde*I and *Xho*I site at the 5′ and 3′ end, respectively. PCR fragments were inserted into the pET21b expression vector (Novagen) between the *Nde*I and *Xho*I site. The recombinant proteins were produced in *Escherichia coli* BL21(LysE) and purified by sodium dodecyl sulfate (SDS)–PAGE using methods described previously [13]. Polyclonal antibody specific for *β6_ca* was raised

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against purified recombinant *β6_ca* according to the procedure described before using a guinea pig [14]. Anti-serum which specifically recognizes the 26 kDa subunit of the 20S proteasome was obtained.

2.4. Purification of the 20S proteasome

Goldfish were purchased from a local supplier and maintained at 15°C until used. The 20S proteasome was purified from immature ovaries of goldfish as previously described [15].

2.5. Electrophoresis and immunoblotting

SDS–PAGE was carried out according to the method of Laemmli [16]. 2D-PAGE (first dimension, NEPHGE; second dimension, SDS–PAGE) was carried out as described by O'Farrell et al. [17] using precast polyacrylamide gel for NEPHGE (Immobiline Dry Strip pH3-10NL, Amersham Pharmacia). Electroblotting and detection using antibodies were conducted as described [15].

2.6. Preparation of GVs and GV-nuclear matrix

GVs were isolated from goldfish oocytes according to Yamaguchi et al. (submitted for publication). Nuclear matrix was prepared from GVs as follows. Isolated GVs (500) were rinsed with 50 mM phosphate buffer pH 7.0 (PB, 500 μl) first, then rinsed with the same volume of nucleic acid digestion buffer (50 mM PB, 10 mM $MgSO_4$, 100 μM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (pAPMSF), pH 7.0) and suspended in the same buffer (500 μl) containing nucleases, 100 units of DNase I and RNase A (Boehringer).

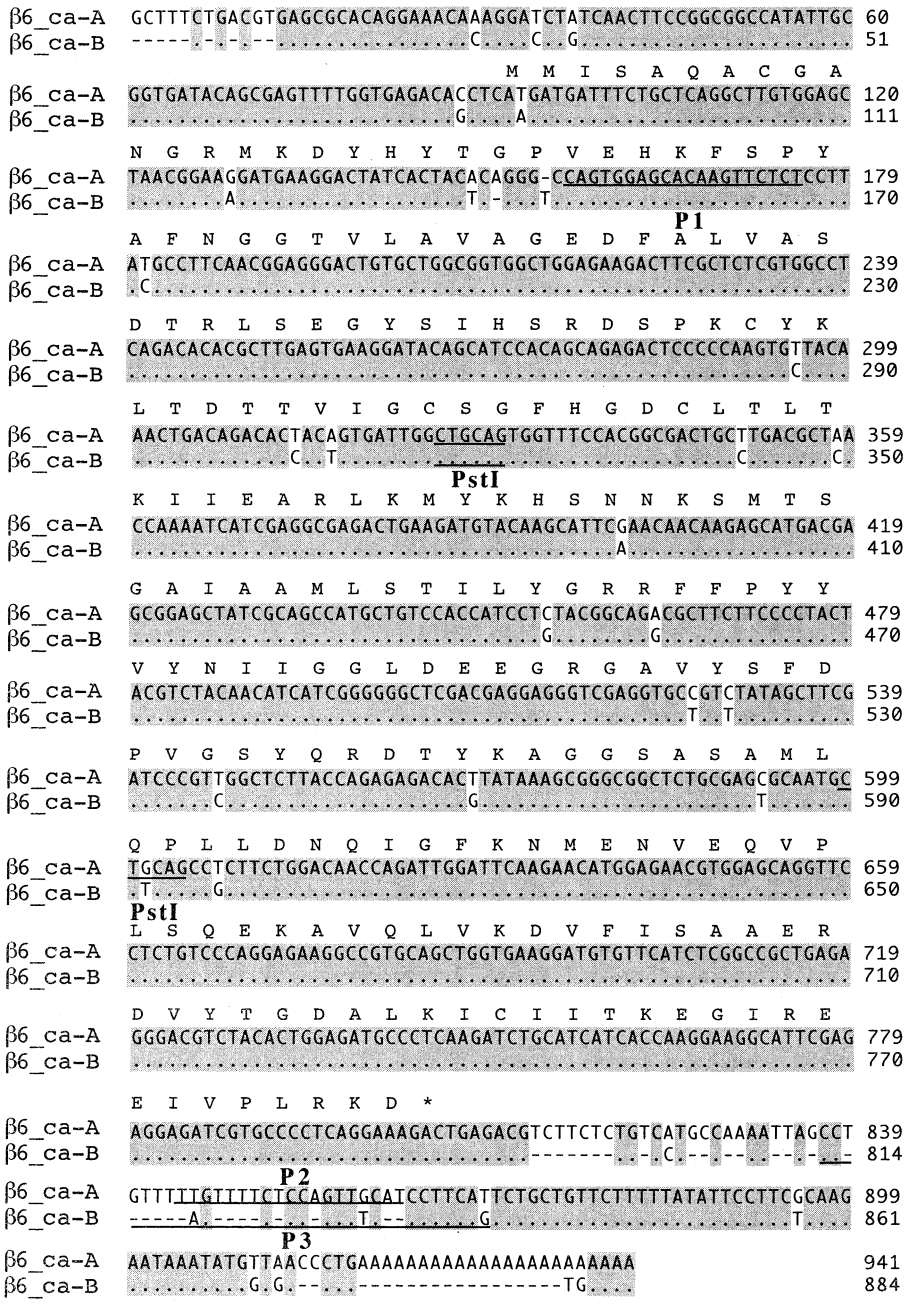

Fig. 1. Nucleotide and deduced amino acid sequences of the *β6_ca* genes of goldfish. The upper letters correspond to the amino acid sequence of *β6_ca*-A. Nucleotide sequences of the two genes are aligned with gaps (hyphens) to maximize matching. Identical nucleotides are indicated by dots. The numbers refer to the nucleotide positions at the end of each line. The positions used to design the specific primers for RT-PCR (P1 to P3) and the *Pst*I site are underlined. The GenBank accession numbers for these sequences are AB035496 (*β6_ca*-A) and AB035497 (*β6_ca*-B).

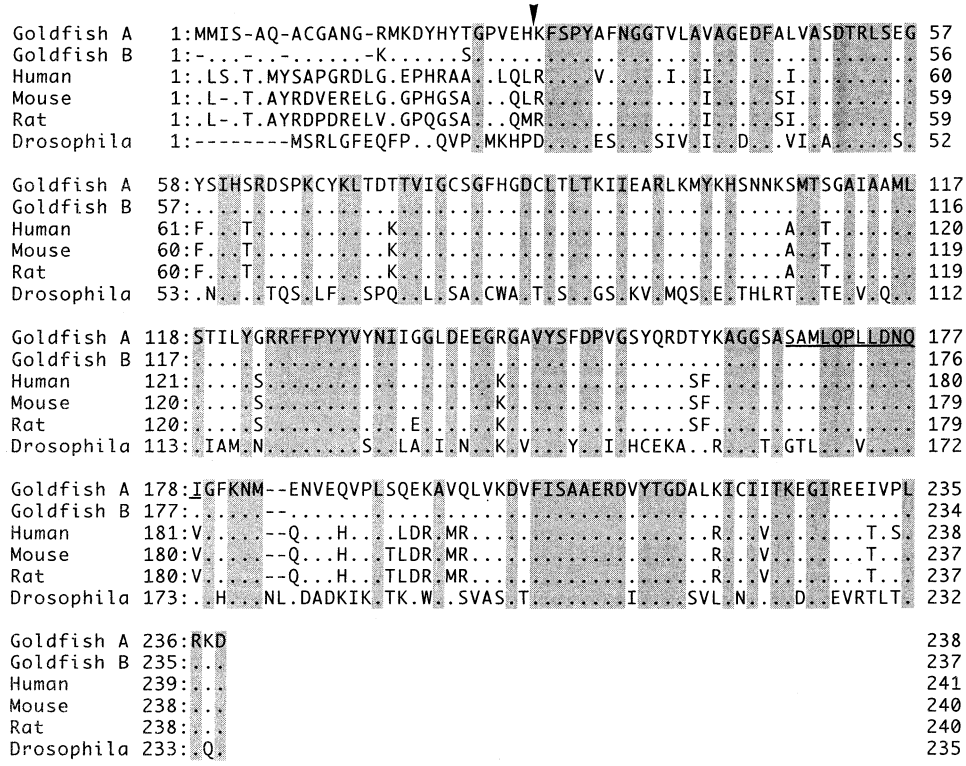

Fig. 2. Comparison of the amino acid sequences for the goldfish, human, mouse, rat and *Drosophila* C5 subunit. Matched sequences are boxed. The numbers refer to the amino acid position at the end of each line. The amino acid sequence of the 26 kDa spot is underlined. The arrow indicates a cleavage site in goldfish, human and rat.

Nuclease digestion was performed with gentle agitation for 1 h at room temperature. After the removal of digestion buffer (little protein was extracted in the digestion buffer as judged by Coomassie staining on SDS–PAGE), GVs were mixed with 1 ml of extraction buffer (PB containing 1 M NaCl, 1% NP-40, 1 mM DTT, 100 μM pAPMSF), then rotated at 4°C overnight. Lamin-enriched nuclear matrix (GVNM) was collected in the pellets by centrifugation at $10\,000\times g$ for 30 min. A second extraction was carried out with the same buffer for 1–2 h rotating at 4°C. After several rinses with 10 mM Tris–HCl (pH 7.4), proteins were extracted from pooled GVNM (equivalent to 2500 GV) with lysis buffer (50 μl; 8 M urea, 0.5% Triton X-100, 1 mM DTT, 0.8% ampholine 3–10) twice. Protein samples were mixed and stored at −80°C until use.

3. Results and discussion

3.1. Isolation and characterization of β6_ca cDNA clones

Five positive clones were isolated from 2×10^5 plaques of the goldfish ovarian cDNA library by screening with PCR-probe. The cDNA clones all contained an insert 1.0 kb long. Further analysis revealed that four clones were identical. Thus, two independent clones were obtained. Both clones were sequenced in their entirety (Fig. 1). The deduced amino acid sequence of each clone contains a sequence of the 26 kDa spot obtained on 2D-PAGE of the nuclear matrix (Fig. 2). As described later, anti-serum raised against the recombinant protein produced from the cloned gene cross-reacted with the spot (Fig. 5). Thus, we concluded that the cDNA isolated in this study encodes the 26 kDa subunit of the goldfish 20S proteasome found in the nuclear matrix. Comparison of the deduced amino acid sequence revealed these molecules are highly homologous to C5 subunits of vertebrates; rat *RC5* (87%), human *HC5* (81%) and mouse *PSMB1* (85%) and relatively less homologous to yeast *Prs3* (47%) and *Drosophila Pros26* (52%) [18–22] (Fig. 2). We concluded that the goldfish cDNA encodes a proteasome subunit corresponding to the C5 subunit. We named these clones *β6_ca*-A and *β6_ca*-B (β6 subunit of *Carassius auratus*) according to a new systematic nomenclature [6]. The number of deduced amino acids and the molecular mass are as follows: 239 amino acids and 26 175 Da for *β6_ca*-A, 238 amino acids and 26 001 Da for *β6_ca*-B. In rat and fish, two types of cDNA encoding the α4 subunit of the 20S proteasome were identified and it was demonstrated that both genes are expressed together in various tissues or at various developmental stages [23,24]. These results suggested that proteasomes are present as a heterogeneous population in various tissues, possibly for the acquisition of diversity of functions of proteasomes. In this study, we identified two types of cDNA encoding the β6 subunit. Then, we investigated whether or not these two types of genes are expressed in various tissues of goldfish by RT-PCR (Fig. 3). DNA fragments of *β6_ca*-A and *β6_ca*-B with expected sizes were amplified using total RNAs of all tissues examined. These results suggest that proteasomes are present as a heterogeneous population in various tissues of goldfish.

3.2. Analysis of subunits of the 20S proteasome in nuclear matrix

Next, we prepared polyclonal antibody against recombinant protein from the cDNA. Anti-serum from the guinea pig clearly cross-reacted with a band of the 26 kDa subunit of 20S proteasome and recombinant *β6_ca* (Fig. 4). The N-terminal amino acid sequence of a spot of *β6_ca* of purified 20S proteasome was XFSPYAFNGGTLAV. This result indicated that goldfish *β6_ca* is autoprocessed at the site corresponding to the human and rat β6 subunit [25,26]. The difference in

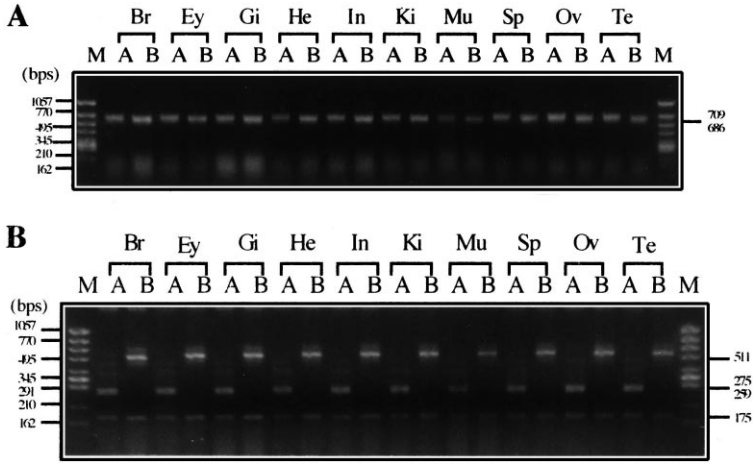

Fig. 3. Electrophoresis of RT-PCR products of *β6_ca*-A and *β6_ca*-B. Amplification of fragments of *β6_ca*-A and *β6_ca*-B was performed as described in Section 2 using total RNA from various goldfish tissues (Br, brain; Ey, eye; Gi, gill; He, heart; In, intestine; Ki, kidney; Mu, muscle; Sp, spleen; Ov, ovary; Te, testis). Lanes A and B indicate the amplification by a pair of primers for *β6_ca*-A and *β6_ca*-B, respectively. To confirm the specificity of the amplification, the PCR products were digested by *Pst*I. By *Pst*I digestion, the PCR products of *β6_ca*-A (709 bp) and *β6_ca*-B (686 bp) were cleaved into three fragments (275, 259 and 175 bp) and two fragments (511 and 175 bp), respectively. The PCR products before (A) and after (B) the *Pst*I digestion were electrophoresed on a 1.5% agarose gel (A) and 4% NuSieve GTG agarose gel (B) and stained with ethidium bromide.

molecular mass between native *β6_ca* and recombinant *β6_ca* coincided with the molecular mass of the processed sequence. The anti-serum also specifically detected a spot of *β6_ca* in 2D-PAGE of nuclear matrix and purified 20S proteasome (Fig. 5). When, the same blot of the 20S proteasome was detected by anti-20S proteasome polyclonal antibody, more than five spots of the 20S proteasome subunits were detected (Fig. 5A). No other spots of 20S proteasome subunits were detected in the blot of nuclear matrix (Fig. 5B). Furthermore, no spots of the 20S proteasome except for the spot of *β6_ca* was observed in the silver stained gel of the nuclear matrix (Yamaguchi and Nagahama, unpublished results). These results suggested that only *β6_ca* remains in the nuclear matrix after the high salt and detergent extraction. It seems that *β6_ca* plays a major role in the anchoring of the 20S proteasome to the nuclear matrix.

Cell cycle-dependent changes in proteasome localization have been widely investigated. Proteasomes are colocalized in nucleus and cytoplasm [27] and accumulate into GV before germinal vesicle breakdown in amphibian oocytes [8]. Cell cycle-dependent accumulation of proteasomes into the nucleus is also observed in ascidian embryos [9]. The dynamics of proteasome distribution in living human culture cells were elucidated using green fluorescent protein tagged with proteasome subunit LMP2 [10]. By this technique, the unidirectional transport of proteasomes from the cytoplasm to nucleus in interphase cells and rapid diffusion during nuclear envelope breakdown in cell division was demonstrated. More recently, localization of the 26S proteasome in yeasts was demonstrated [11,12]. According to the results, the majority of the 26S proteasome is localized at the nuclear periphery in yeast. We have sequenced 14 spots of 2D-PAGE whose molecular mass ranges from 38 to 70 kDa. A spot of 52 kDa gave a sequence highly homologous to that of TBP-1, one of the regulatory subunit of the 26S proteasome (Yamaguchi and Nagahama, unpublished results). The results suggest that TBP-1 may contribute to anchoring the 19S regulatory complex to the nuclear matrix as β6 for the 20S proteasome. It seems possible that the 26S proteasome is localized by the specific components of each subcomplex in the nuclei. The question remains why only β6 is capable of binding to the nuclear matrix. One of the non-catalytic subunits, β6 has no known motif sequence but has a specific insertion segment at C-terminal region [6]. There is a possibility that the specific segment is involved in binding to the nuclear matrix.

In the present study, we identified a candidate for a proteasomal subunit which affects the concentration of proteasomes in the nucleus. Identification of the site in the nuclear matrix of interaction with the β6 subunit, may facilitate understanding of the translocation into and the concentration of proteasomes in the nucleus.

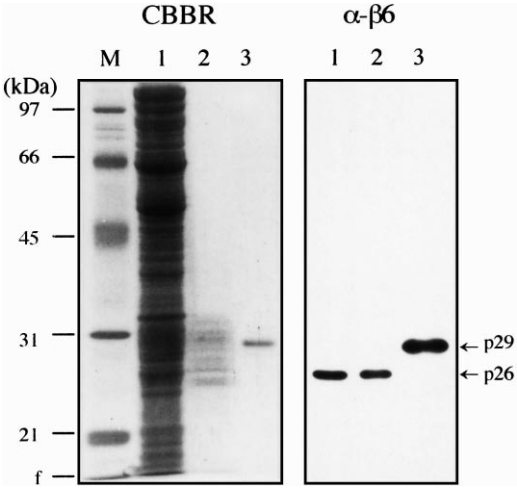

Fig. 4. Immunoblotting of the purified 20S proteasome and recombinant *β6_ca*. Cytosol from goldfish immature oocytes, purified 20S proteasome and recombinant *β6_ca* were electrophoresed under denaturing conditions (12.0% gel) and stained with Coomassie Brilliant Blue (CBBR) or immunostained with anti-recombinant *β6_ca* anti-serum after electroblotting. Protein bands that cross-reacted with anti-serum (p26 and p29) are indicated by arrows. Molecular masses of standard proteins are indicated at the left.

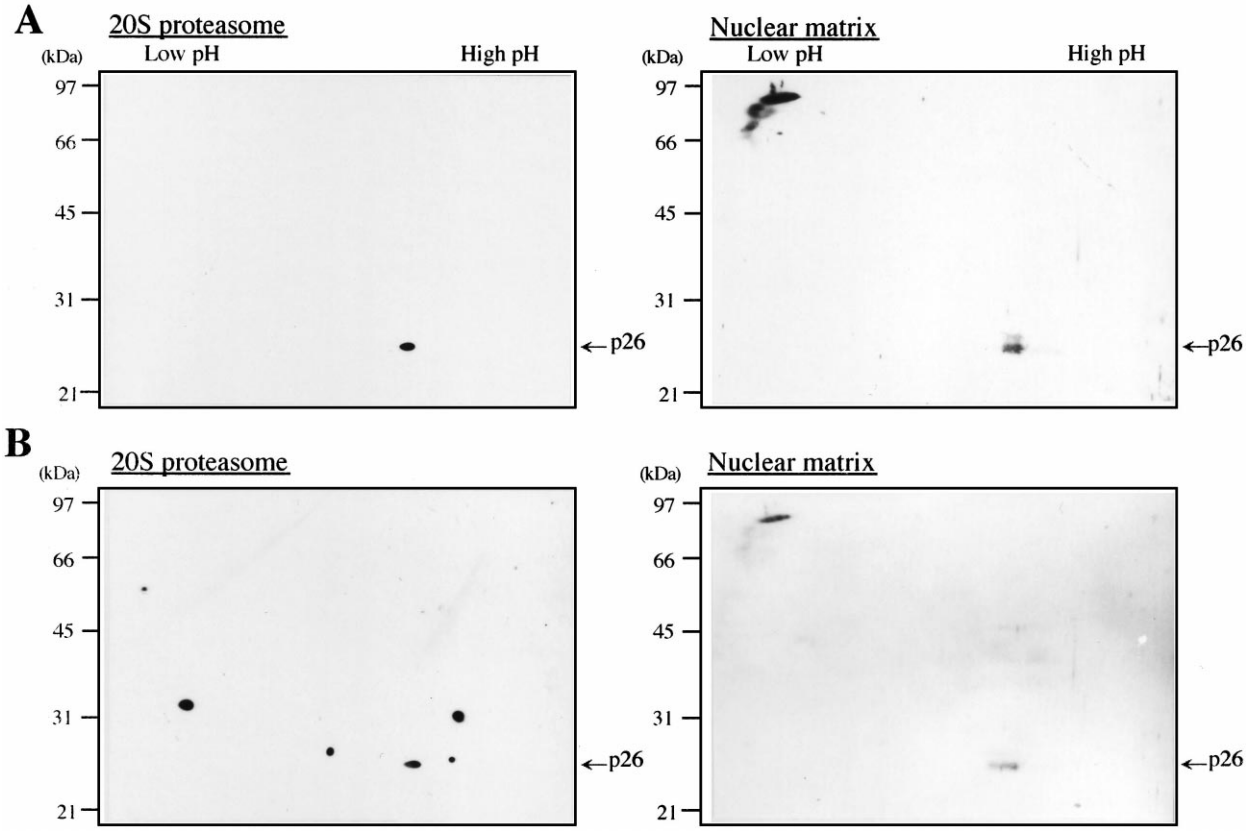

Fig. 5. 2D-PAGE analysis of the 20S proteasome and nuclear matrix. The 20S proteasome from immature oocytes and the nuclear matrix fraction were subjected to 2D-PAGE followed by immunostaining by anti-recombinant *β6_ca* (A) and anti-*Xenopus* 20S proteasome polyclonal antibody [14] (B). The spot of 26 kDa which cross-reacted with anti-recombinant *β6_ca* is indicated by an arrow.

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