ISOLATION AND CHARACTERIZATION OF PARVALBUMIN FROM CHICKEN LEG-MUSCLE

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1. Introduction

Parvalbumins are characterized by their high solubility in water, low molecular weight of approximately 12 000, acidic isoelectric point, heat stability, high phenylalanine content relative to tyrosine and tryptophan and by their specific binding of 2 Ca^{2+}-ions/molecule [1].

Parvalbumins have recently been found in muscle from higher vertebrates [2], but characterized in detail only in lower vertebrates [3–6] and rabbit muscle [2,7,8]. No certain physiological function can be ascribed to them as yet.

The myogen of chicken leg-muscle contains several acidic, low molecular weight components:

(a) A parvalbumin-like protein (PVlP) with an apparent molecular weight of 12 000 and an affinity for Ca^{2+} as recently described by us [9].

(b) Two other components with similar properties but with different migration rates on 'native' polyacrylamide gel electrophoresis and with distinct amino acid compositions (to be published).

(c) A parvalbumin, which will be described here.

The present report describes an isolation procedure for parvalbumin from chicken leg-muscle utilizing properties such as heat and acid stabilities. The purified component is compared to the rabbit protein, the only other parvalbumin from vertebrate muscle fully characterized up to now [7,8] and to PVlP [9], the latter having been extracted under identical conditions as parvalbumin and copurified through several steps of purification.

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2. Experimental

Chickens were obtained from Kneuss, Mägenwil, Switzerland.

2.1. *Electrophoretic procedures*

Polyacrylamide gel electrophoreses were carried out either in Tris–glycine buffer, pH 8.3, on 7.5% gels [10] or in the presence of 0.1% sodium dodecyl sulphate on 10% gels [11].

2.2. *Determination of molecular weight*

The molecular weight of parvalbumin was estimated by dodecyl sulphate–polyacrylamide gel electrophoresis by direct comparison with PVlP (9) and cytochrome *c* assuming a molecular weight of 12 000 for these two proteins.

2.3. *Absorption spectra and Ca^{2+}-content*

The ultraviolet spectrum was obtained with a Perkin-Elmer 124 spectrophotometer and the Ca^{2+}-content measured on a Perkin-Elmer atomic absorption spectrometer, Model 303, using 'spec pure' salts (Merck) as standards.

2.4. *Protein concentration*

Protein concentrations were measured routinely

either according to Lowry et al. [12] or by using the absorption $A^{1\%}_{258\ nm}$ for the purified parvalbumin.

2.5. *Amino acid analysis*

The amino acid analysis was performed on a Beckman Model 121 analyser. The samples were hydrolyzed in 6 N HCl for 24 h, 48 h and 72 h at 110°C in sealed ampoules evacuated after several rinsings with pure nitrogen.

2.6. *Antibody preparation*

Antibodies against PVlP from the chicken were elicited in the rabbit as described earlier [9].

2.7. *Isolation and characterization of PVlP and β-actinin*

This is described elsewhere [9,13].

3. Results

3.1. *Purification of chicken parvalbumin*

Parvalbumin was identified during the purification by gel electrophoresis in the presence of dodecyl sulphate. The parvalbumin migrates as a major band close to the dye front, identical to PVlP or cytochrome *c*. In the final stages of purification it also could be characterized, and differentiated from PVlP, by its unusually high 260–280 nm absorbance ratio and its characteristic ultraviolet spectrum.

All steps were carried out at 4°C unless stated otherwise. All solutions contained 0.1 mM phenylmethylsulfonylfluoride and 30 units/ml trasylol (Bayer) as protease inhibitors.

Fresh leg-muscle (1.6 kg) was ground in a meat grinder and 2.5 vol. 4 mM EDTA, pH 7, was added. The suspension was homogenized in a Braun mixer for 1 min at full speed and the homogenate centrifuged for 30 min at 14 000 × *g*. The supernatant was then adjusted to pH 7 with 17% ammonium hydroxide and heated to 70°C for 30 min under constant stirring. The suspension was immediately cooled to 10°C and filtered through a sintered glass funnel (under slight vacuum). Proteins were precipitated by addition of one-sixth vol. cold 100% (w/v) trichloroacetic acid to the clarified solution. The pellet, collected by centrifugation, was dissolved in 200 ml 10 mM sodium acetate, pH 5.7, containing additionally 1 mM β-mercaptoethanol and 0.1 mM $CaCl_2$ and dialyzed against the same solution with 1 change of dialyzing buffer. The dialyzed solution was clarified by centrifugation (20 min at 15 000 × *g*) and the 'crude parvalbumin' fraction directly applied to a DEAE-cellulose column (2.8 × 35 cm) equilibrated with the same buffer (fig.1).

After washing until the absorbance of the eluant at 280 nm had dropped below 0.02, the column was developed with a linear gradient from 10–500 mM sodium acetate, pH 5.7. Parvalbumin was not retained on the column and appeared in the breakthrough-peak, well separated from PVlP which was eluted at 40–60 mM salt (peak I) (fig.1). β-Actinin, with a molecular weight of 65 000, eluted at 120–150 mM salt (peak II); its properties are described elsewhere [13]. Fractions 35–80, containing almost pure parvalbumin, were pooled, dialyzed against H_2O, and lyophilized. The powder was dissolved in 1.5 ml 10 mM imidazole–HCl, pH 6.8 and passed through a Sephadex G-75 column (3 × 100 cm) in the same buffer. Fractions containing parvalbumin were pooled, dialyzed extensively first against H_2O and then against 2 mM Tris–HCl, pH 7, and finally applied to a second DEAE-cellulose column (1 × 20 cm) equilibrated with the same buffer. Under these conditions parvalbumin is retained on the column. A linear salt-gradient of 0–300 mM NaCl was applied and homogeneous parvalbumin was eluted at 20–30 mM salt concentration. A contaminant with a molecular weight clearly below 12 000 could be separated and was eluted at 10–20 mM salt.

A summary of the purification is given in table 1.

Approximately 10–20 mg parvalbumin was obtained from 1 kg muscle. This corresponds to approximately 20% of the amount of parvalbumin isolated from rabbit muscle [8].

3.2. *Criteria of purity and determination of the molecular weight*

Parvalbumin was pure as judged by polyacrylamide gel electrophoresis in the presence (fig.2A) or absence (fig.3A) of sodium dodecyl sulphate. Judged from the direct comparison of SDS–gel electropherograms (PVlP, fig.2B; cytochrome *c*, fig.2C) an identical molecular weight of about 12 000 for parvalbumin was obtained. However,

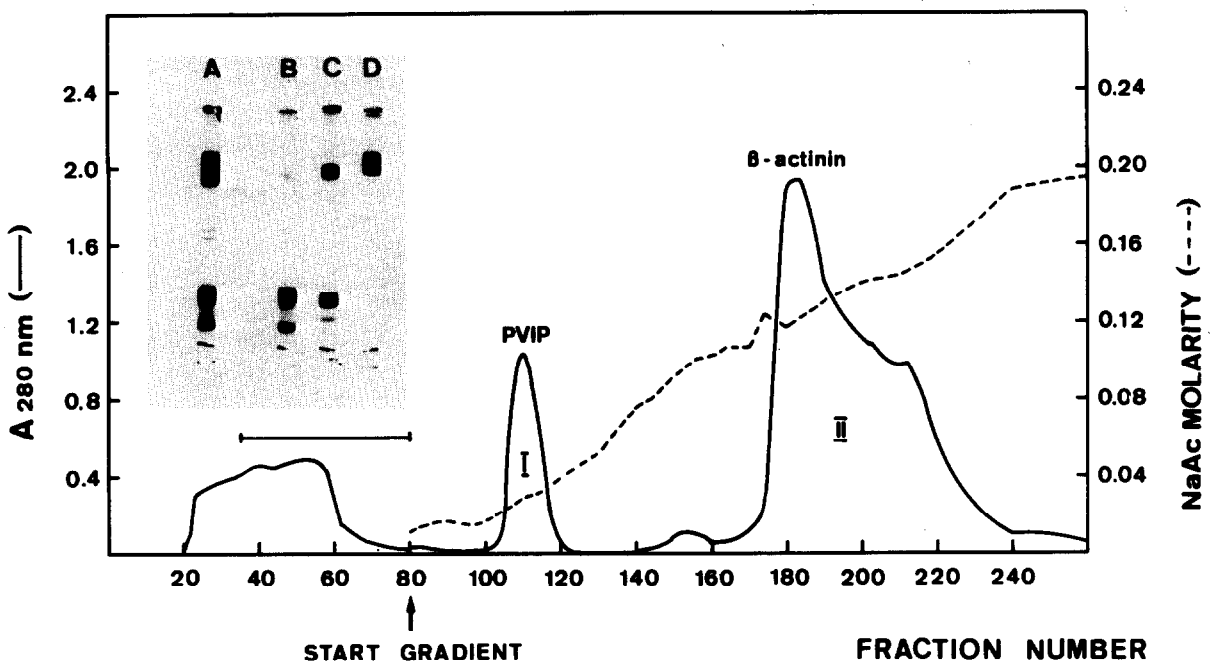


Fig. 1. Chromatography of the 'crude parvalbumin' fraction after precipitation with trichloroacetic acid, on a DEAE-cellulose column. Protein, 900 mg, dissolved in 10 mM sodium acetate, pH 5.7, was loaded. The column was washed and a linear gradient (10–500 mM salt) was applied. Polyacrylamide gels (10%) in the presence of 0.1% dodecyl sulphate are shown corresponding to (A) load of protein (100 μg), (B) breakthrough, pooled fractions 35–80 (120 μg), (C) peak I, fraction 110 (25 μg) and (D) peak II, fraction 180 (30 μg).

Table 1
Isolation of parvalbumin from chicken muscle

Step		Volume (ml)	Protein (mg/ml)	Total protein (mg)	Yield (%)
1	4 mM EDTA extract	3680	5.4	19 872	100
2	after heat step and filtration	3465	0.64	2218	11.2
3	after TCA step ('crude parvalbumin' fraction)	225	4.0	900	4.5
4	1. DE-52 effluent	230	0.64	147	0.7
5	Sephadex G-75 effluent	100	0.42	42	0.2
6	2. DE-52 effluent (pure parvalbumin)	14	1.33	18.6	0.1

At Steps 1–5 the protein concentrations were measured by the procedure of Lowry et al. [12]. At Step 6, the absorption coefficient $A^{1\%}_{258\ nm}$ of 1.85 calculated for the purified parvalbumin was used. Fresh time, 1.6 kg, was used in this preparation.

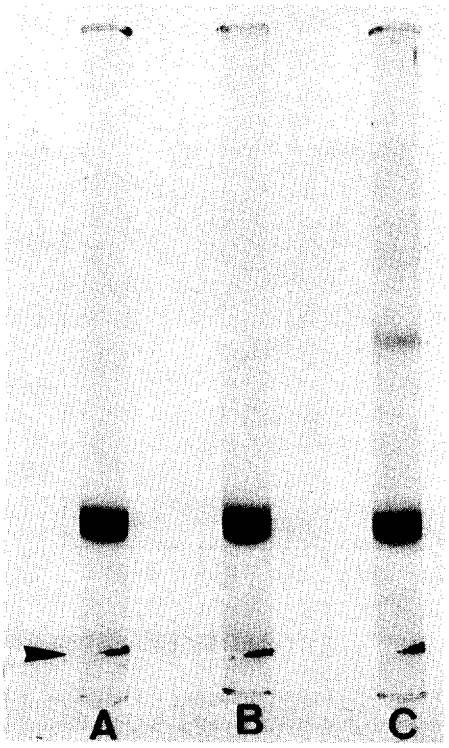


Fig.2. Polyacrylamide gels (10%) in the presence of 0.1% sodium dodecyl sulphate of (A) chicken parvalbumin (10 μg), (B) PVlP (10 μg) and (C) cytochrome c (20 μg) (mol.wt 12 000) as marker protein. Migration is from top to bottom. Arrow indicates dye front.

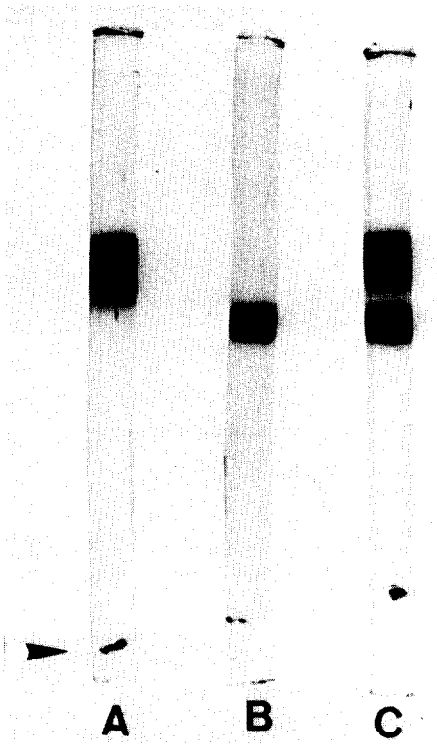


Fig.3. 'Native' polyacrylamide gels (7.5%) of (A) chicken parvalbumin (30 μg), (B) PVlP (10 μg) and (C) mixture of both proteins (10 μg each).

parvalbumin showed a considerably slower migration rate compared to PVlP in 'native' gel electrophoresis (fig.3), indicating a lower negative net charge of this protein.

3.3. *Calcium analysis*

The Ca^{2+}-content of chicken parvalbumin was determined by atomic absorption using conditions identical to those applied for PVlP [9] or the calcium-binding subunit of troponin (TN-C) of the pacific dog fish [6]. An average of 2 ± 0.2 gatoms Ca^{2+}/mol parvalbumin was found, a result in agreement with the Ca^{2+}-content of all other parvalbumins.

3.4. *Amino acid composition*

The amino acid composition of the chicken parvalbumin is illustrated in table 2 and is compared to that of the rabbit protein and to PVlP. Almost identical values are obtained when the chicken protein is compared to the rabbit parvalbumin; this includes the high content of phenylalanine (9 residues) found for most parvalbumins. However, the chicken protein has one tyrosine in additon. These values are also very similar to those obtained from lower vertebrates, including the pacific dog fish [6], frog [14], carp [4], hake [3] and ray [5]. Considerable differences are seen in the content of threonine, alanine, phenylalanine, cysteine and tryptophan when compared to the PVlP [9].

The absorption coefficient $A^{1\%}_{258\ nm}$ of 1.85 was calculated from the amino acid composition [15]. The partial specific volume ($\bar{v}$) was 0.74 ml/g at 20°C, applying a temperature correction factor $d\bar{v}/dt$ of $0.0005 \times g^{-1}$ [16].

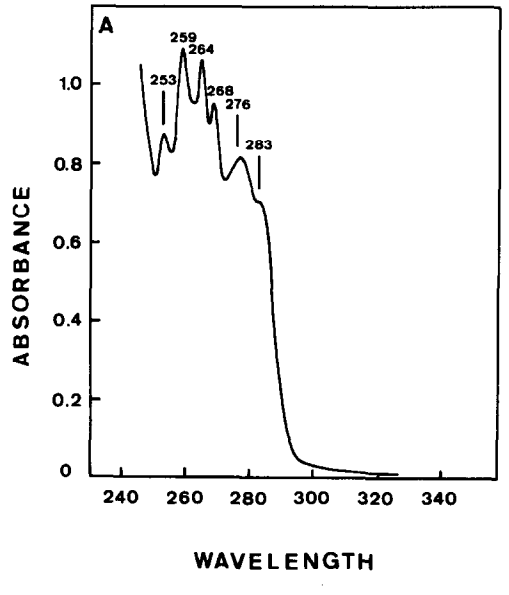
Table 2
Amino acid composition of chicken parvalbumin

Amino acid[a]	Residues/molecule[b]		
	Chicken parvalbumin	Rabbit parvalbumin[c]	Parvalbumin-like protein from chicken
Lysine	13.2	16	12.3
Histidine	1.1	2	2.3
Arginine	2.2	1	3.4
Aspartic acid	11.8	12	11.6
Threonine[d]	4.8	5	13.0
Serine[d]	7.6	8	5.8
Glutamic acid	11.6	13	11.5
Proline	2.6	1	1.9
Glycine	9.9	9	9.2
Alanine	12.5	11	6.9
Valine	7.7	5	10.8
Methionine	2.8	3	2.3
Isoleucine	3.9	6	3.2
Leucine	7.8	9	9.9
Tyrosine	1.1	0	1.6
Phenylalanine	9.4	9	4.7
Cysteine[e]	0	0	2.0
Tryptophan[f]	0	0	1.1

[a] Average of 24 h, 48 h and 72 h hydrolysis
[b] Assuming a mol.wt 12 000
[c] Obtained from sequence data [7]
[d] Extrapolated to zero time of hydrolysis
[e] Determined as cysteic acid [21]
[f] Measured spectrophotometrically [22]

3.5. *Absorption spectra*

The absorption spectrum of chicken parvalbumin is illustrated in fig.4A. It can be seen that the chicken protein displays the same vibrational structures as rabbit parvalbumin in the 260 nm region which is due to a high phenylalanine to tyrosine ratio and a complete lack of tryptophan. The additional shoulder at 278 nm of the chicken protein (fig.4A) when compared to the rabbit component (fig.4B) results from the presence of 1 additional tyrosine residue. Considerable differences compared to the PVIP (fig.4C) are obvious. The differences result mainly from the presence of 1 tryptophan besides 1–2 tyrosine in the PVIP molecule. The 260 nm to 280 nm absorbance ratio of parvalbumin is 1.35.



3.6. *Immunological properties*

Although parvalbumin and PVIP show similarities in solubility, molecular weight and affinity for

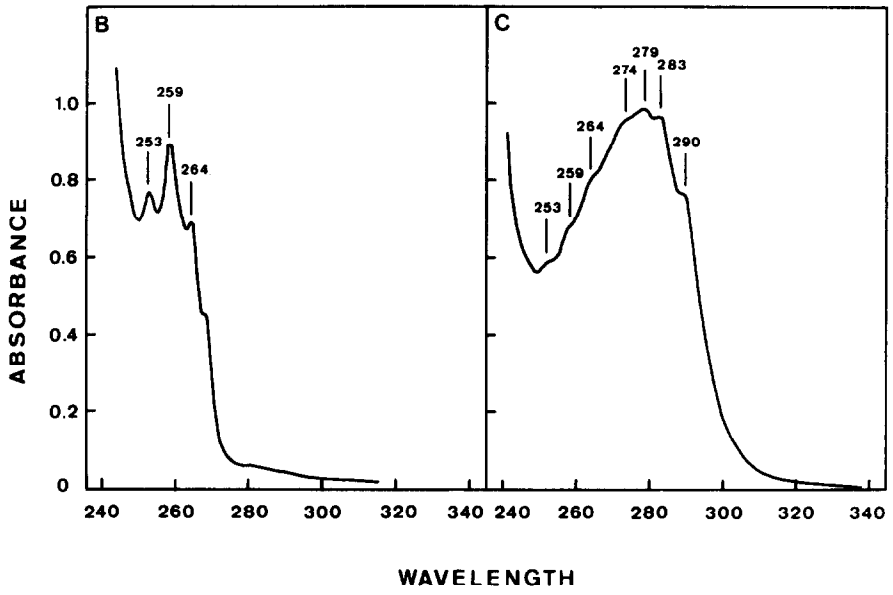


Fig.4. Ultraviolet absorption spectra of (A) chicken parvalbumin, (B) rabbit parvalbumin and (C) PVIP in 2 mM Tris–HCl, pH 7. The protein concentrations were 1.5; 6.6 and 1.3 mg/mg, respectively.

Ca^{2+}-ions, they are, however, immunologically distinct proteins. This is demonstrated on Ouchterlony plates (fig.5). Anti-PVIP serum neither cross-reacted with parvalbumin nor with β-actinin from the chicken.

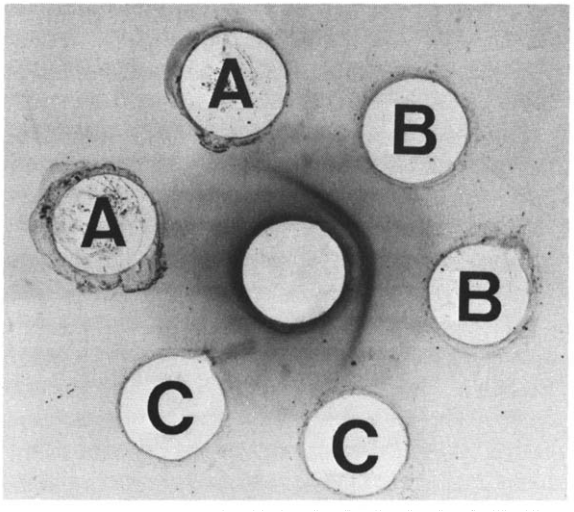


Fig.5. Ouchterlony double immunodiffusion test of anti-PVIP (center well) against (A) purified parvalbumin, (B) PVIP and (C) β-actinin. Staining of the proteins was performed in 0.5% amidoblack, 45% methanol, 10% acetic acid.

4. Discussion

These findings show that parvalbumins, present in sizable amounts in the muscle of vertebrates, are not restricted to lower vertebrates but have apparently been conserved throughout evolution. This suggests that parvalbumins might play an essential physiological role not detected until now.

The parvalbumin isolated from chicken leg-muscle is very similar to the rabbit protein in a number of properties such as molecular weight, amino acid composition, unusual ultraviolet absorption spectrum and Ca^{2+}-content [2,7,8]. The much lower tissue concentration of only 10–20% of the amount found in rabbit muscle and the presence of 1 tyrosine residue/molecule are, however, distinct features to the rabbit counterpart.

The chicken muscle contains, besides parvalbumin, several other components with molecular weights of approximately 10 000–12 000, of which only a calcium-binding parvalbumin-like protein (PVIP) has been described in detail [9]. It was found that PVIP (a) accumulates in differentiating primary muscle-cell cultures after 120 h, approximately together with myosin and actin and (b) is partially

bound to the I-band region of isolated myofibrils. This result suggested to us that PVlP could well be linked in some way to the contractile process.

This was also suggested for the parvalbumins based on the findings that parvalbumin [17] and TN-C [6,18] show comparable Ca^{2+}-affinities and a high degree of structural homology [19,20].

Therefore similar investigations, as done for the PVlP molecule [9], are now in progress in hope of getting more information about the role of parvalbumins in muscle.

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