GLYCOGEN DEBRANCHING ENZYME FROM CHICKEN PECTORALIS MUSCLE

Comparison with a 165 000 mol. wt myofibrillar protein

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1. Introduction

M-line proteins have been isolated by several investigators using various procedures [1–5]. Of these, low salt extraction is particularly valuable since it removes the M-line rather specifically [1–4]. Electrophoretic analysis of the low salt extract (5 mM Tris–HCl (pH 8)) showed three major protein bands on $NaDoSO_4$ gel electrophoresis, with subunit molecular weights in the range of 40 000, 100 000 and 165 000. The smallest of these proteins was identified as MM-creatine kinase [6] and the 100 000 mol. wt component was shown to be muscle glycogen phosphorylase [7,8]. Since glycogen debranching enzyme from rabbit muscle has mol. wt 166 000 [9] and since it is known to occur in a complex with phosphorylase and glycogen [9,10] we suggested [7,11–14], that the 165 000 protein from myofibrils might be debranching enzyme. Consistent with this idea we subsequently reported that the low salt extract contained debranching activity [11–14].

More recently, it was shown [8] that myofibrillar extracts contain in fact two proteins with identical molecular weights in the range of 165 000 but with distinct sedimentation coefficients. One component, the 5 S protein, could be shown to be bound to the M-region of isolated myofibrils. The other protein (7 S) was found to have a different location within myofibrils and to exhibit properties similar to debranching enzyme. However, identity of the 165 000 (7 S) myofibrillar protein with debranching enzyme has never been unambiguously demonstrated. The present study was initiated in order to eliminate this shortcoming.

This work reports the first isolation of glycogen debranching enzyme (1,4-glucan-4-glucosyltransferase plus amylo-1,6-glucosidase) from chicken pectoralis muscle in a homogeneous form and directly compares its physico-chemical properties with those of the homologous 165 000 (7 S) myofibrillar protein. A comparison is also made with the glycogen debranching enzyme from rabbit. So far, only the enzymes from rabbit [9], dog fish [15] and baker's yeast [16] have been characterized in detail. Based on these investigations we conclude that the 165 000 (7 S) myofibrillar protein is likely to represent glycogen debranching enzyme.

2. Materials and methods

2.1. *Buffer solutions*

Buffer A contained 5 mM Tris–HCl, 1 mM EDTA, 14 mM β-mercaptoethanol (pH 7.2) and buffer B, 50 mM glycerol-1-phosphate, 2 mM EDTA, 1 mM dithiothreitol (pH 7). All solutions contained in addition 0.1 mM phenylmethylsulfonylfluoride and 10^{-6} M pepstatin (Sigma).

2.2. *Purification of debranching enzyme*

The enzyme was isolated from the breast muscle

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Abbreviation: $NaDoSO_4$, sodium dodecyl sulfate

of freshly killed chicken essentially as described for the rabbit enzyme [9], but with modifications indicated in the text.

2.3. *Isolation of the 165 000 mol. wt (7S) myofibrillar protein*

This was performed in our laboratory by E. Strehler according to published methods [8].

2.4. *Polyacrylamide gel electrophoresis*

This was performed either with [17,18] or without [19] $NaDoSO_4$ as described. Two dimensional gels were run according to [20].

2.5. *Ultracentrifugation*

This was carried out in a Spinco Model E analytical ultracentrifuge. Sedimentation velocity experiments (at 3.3 mg protein/ml) were carried out in buffer B (see above) having a relative viscosity of 1.037 and a density of 1.005 g/ml at 20°C [21]. High speed sedimentation equilibrium experiments were performed in the same buffer. The partial specific volume was 0.736 (calculated from the amino acid composition). The temperature correction factor dv/dt = 0.0005 ml $.g^{-1}.K^{-1}$ was applied [22].

2.6. *Absorption coefficient*

$A^{1\%}_{280}$ was determined by the method in [23] and found to be 17.8 for debranching enzyme.

2.7. *Protein concentration*

This was measured according to [24] or, for the purified enzyme, by using $A^{1\%}_{280}$.

2.8. *Amino acid analysis*

This was performed on a Beckman 'Multichrom' analyser. The samples were hydrolysed in 6 N HCl for 24, 48 and 72 h at 110°C. Cysteine was determined after oxidation with performic acid [25] and tryptophan was measured spectrophotometrically [26].

3. Results

3.1. *Isolation of debranching enzyme*

The enzyme was precipitated at 41% ammonium sulfate. The pellet, obtained by centrifugation, was dissolved in buffer A, dialysed, and applied to a DEAE-cellulose column (40 × 3 cm) equilibrated in buffer A. The column was developed with a linear salt gradient (0–0.2 M NaCl) and the debranching enzyme was eluted at 60 mM salt. The eluate was dialysed against buffer A and then passed over a Sepharose–$NH(CH_2)_4NH_2$ column, equilibrated in the same buffer. The column was washed successively with buffer A, buffer A + 50 mM NaCl, and buffer A + 500 mM salt. Most of the phosphorylase was eluted at 50 mM NaCl. The debranching enzyme, on the

Table 1
Purification of chicken muscle debranching enzyme

Step	Volume (ml)	Protein (mg)	Activity (U)	Spec. act. (U/mg)	Purification	Yield (%)
1. Extract	1750	30 625	4725	0.15	1	100
2. 41% $(NH_4)_2SO_4$ precipitation and dialysis	250	4250	2000	0.47	3.1	42
3. DEAE-cellulose (pH 7.2)	200	820	2080	2.54	17	44
4. Sepharose $-NH(CH_2)_4NH_2$	43	3.7	303	82	546	6.4

800 g of muscle were used in this preparation. At steps 1-3 protein was measured according to [24]. In step 4 the absorption coefficient $A^{1\%}_{280\ nm}$ of 17.8, found for the purified enzyme, was used

other hand, remained attached to the column and was eluted with buffer A + 500 mM salt. It was stored at 4°C in buffer B until used for characterization. A summary of the purification is given in table 1.

Fig. 1. Polyacrylamide (8%) gel electrophoresis in the presence of $NaDoSO_4$. (a) Chicken debranching enzyme. (b) Rabbit debranching enzyme. (c) Mixture of both. (d) M_r = 165 000 (7 S) protein. (e) Mixture of M_r = 165 000 protein with chicken debranching enzyme. About 2 μg of each protein were applied. Migration is from top to bottom, arrow indicates dye front.

3.2. *Activity of debranching enzyme*

Two independent methods were used to determine the activity of the enzyme:

Method 1 makes use of the slight reversibility of the debranching enzyme reaction [27] by following the incorporation of [^{14}C]glucose into glycogen [9,28]. This procedure was used for routine determination of enzyme activity during purification. Using this method, a specific activity of 82 units/mg was obtained for the chicken enzyme compared

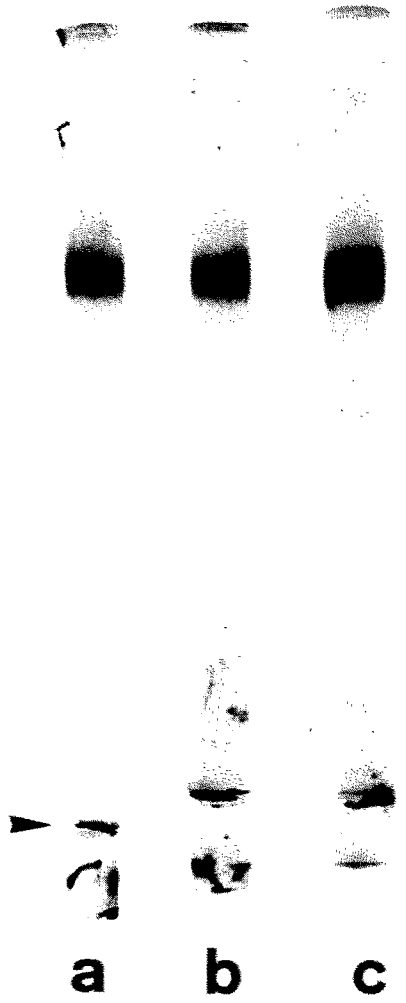

Fig. 2. Polyacrylamide (7.5%) gel electrophoresis in the absence of $NaDoSO_4$. (a) Chicken debranching enzyme. (b) M_r = 165 000 (7 S) protein. (c) Mixture of both. Approx. 10 μg of each protein were applied. Migration is from to to bottom, arrow indicates dye front.

with 107 units/mg found for the rabbit protein [9] measured under identical conditions.

Method 2 is a titrimetric assay [9,29] which exploits the difference in pK between the second ionization of glucose-1-phosphate (pK = 6.1) and inorganic phosphate (pK = 6.8). Identical activities for the chicken and rabbit enzymes were obtained by this method.

3.3. *Homogeneity and molecular weight of chicken debranching enzyme*

Purified debranching enzyme migrated as a single protein band on polyacrylamide (8%) slab gel electrophoresis in the presence of $NaDoSO_4$ (fig.1a) as well as on 7.5% gels in the absence of detergent (fig.2a). When the purified protein was subjected to the two-dimensional gel electrophoresis according to [20], a single protein spot was obtained (not shown). The subunit molecular weight of the chicken debranching enzyme was estimated from $NaDoSO_4$ electrophoresis by direct comparison with the rabbit enzyme (fig.1a–c) for which mol. wt 166 000 was reported [9]; both enzymes migrated identically (fig.1a,b) and equal mixtures revealed only a single band (fig.1c). Thus chicken debranching enzyme has a mol. wt ~166 000.

Further evidence for homogeneity was obtained from the centrifugation studies described below.

3.4. *Comparison of chicken debranching enzyme with homologous 165 000 mol. wt (7 S) myofibrillar protein*

Debranching enzyme and the myofibrillar protein had identical mobilities on gel electrophoresis with (fig.1d,e) and without (fig.2) $NaDoSO_4$, as well as on two dimensional O'Farrell gels [20] (not illustrated).

Sedimentation velocity experiments of debranching enzyme showed a single peak with a sedimentation coefficient ($s_{20,w}$) of 8.2 ± 0.1 S. This compares favorably with the $s_{20,w}$ = 8 S obtained with the myofibrillar protein under identical conditions. Similarly, when the molecular weight of the native enzyme was determined by high speed equilibrium ultracentrifugation the purified protein showed a homogeneous distribution of material. The weight, average molecular weight, M_w, obtained was 155 000, in agreement with the values reported for the myofibrillar protein (166 000) [8].

Table 2
Amino acid composition of chicken debranching enzyme (expressed in residues/10^5 g)

Amino acid[a]	Chicken debranching enzyme	Myofibrillar protein 165 000 mol. wt (7 S) from chicken muscle[b]
Aspartic acid	85.0	99
Threonine[c]	43.5	42
Serine[c]	50.6	51
Glutamic acid	92.0	99
Proline	56.0	54
Glycine	70.1	72
Alanine	56.8	60
Valine[d]	63.4	61
Metionine	13.1	21
Isoleucine[d]	56.1	51
Leucine	77.5	85
Tyrosine	44.0	38
Phenylalanine	46.7	38
Histidine	37.6	36
Lysine	47.8	50
Arginine	59.6	53
Cysteine[e]	26	–
Tryptophan[f]	26	–

[a] Average from 3 times of hydrolysis (24, 48, 72 h)
[b] From [8]
[c] Obtained by extrapolation to t = 0
[d] Obtained from 72 h hydrolysis time
[e] Measured after performic acid oxidation [25]
[f] Determined spectrophotometrically [26]

The amino acid composition of chicken debranching enzyme is given in table 2 and is compared with that of homologous 165 000 (7 S) myofibrillar protein. The compositions of both proteins are in good agreement for most residues except for the methionine content, which was lower for the debranching enzyme. However, in view of the known susceptibility of this amino acid to destruction too much weight should not be attributed to this discrepancy.

4. Discussion

The results presented above show that homogeneous chicken debranching enzyme is very similar if not identical, to a 165 000 (7 S) myofibrillar protein by enzymological and physico-chemical criteria. Thus the 7 S myofibrillar protein is associated with debranching activity and both proteins are identical

by molecular weight, sedimentation behaviour, charge and isoelectric point.

Earlier immunofluorescent studies [8] have revealed that, of the two 165 000 mol. wt myofibrillar proteins, only the 5 S component is bound to the M-line, whereas the 7 S protein is located in the I-band. This region also contains substantial amounts of glycogen phosphorylase [7,8], which is known to be intimately associated with glycogen debranching enzyme and their common substrate, glycogen [9,10]. The existence of such a complex in the I-band of myofibrills and the similar location of the 7 S protein, likely to be identical with debranching enzyme, are therefore in good agreement.

Coming back to the question of M-line proteins, the revised list now contains only two protein species, namely MM-creatine kinase and the 165 000 mol. wt (5 S) myofibrillar protein. Although two additional proteins, glycogen phosphorylase and the 165 000 mol. wt (7 S) or debranching enzyme, can be extracted from myofibrils under conditions where only the M-line is removed (5 mM Tris–HCl, (pH 8)) these cannot be considered as true M-line constituents. Presumably these procedures also remove some glycogen with associated enzymes from the I-region of the myofibrils. In this connection it is interesting to note that when isolated myofibrils are incubated with α- or β-amylases, which would be expected to degrade the glycogen in the myofibrils, a release of glycogen-bound enzymes such as phosphorylase, debranching enzyme, glycogen synthetase and phosphorylase kinase into the supernatant has been observed (unpublished experiments). This is also supported by the findings [15] that after extraction and extensive washing of dog fish skeletal muscle, 20% of debranching activity remained with the contractile proteins and could only be solubilized with high salt.

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