Project 505.7031

Het bepalen van het vleesgehalte in vleesprodukten (en de herkomst van dit vlees gerelateerd aan de diersoort) met behulp van actine

(drs J.M.P. den Hartog)

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FAST PROTEIN LIQUID CHROMATOGRAPHY - GEL FILTRATION OF ACTIN AND ITS QUANTITATIVE ANALYSIS BY 3 METHYL-L-HISTIDINE

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Fast Protein Liquid Chromatography of Actin by SDS-Gel filtration

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Fast Protein Liquid Chromatography von Actin mittels SDS-Gel filtration.

Zusammenfassung

Gel filtration wird seit Jahren für die Trennung von Proteinen angewendet. Die konventionelle Gel filtrationstechnik ist sehr langsam und limitiert die Zahl der Experimenten.
Mit der Introduktion der FPLC ist es möglich eine gleiche Zahl von Experimenten in einem Tag, statt einer Woche, auszuführen.
Die FPLC-Gel filtration wurde für die Isolation von Actin aus einem Gemisch von Myofibrillarproteinen und aus Muskelfleisch angewendet.
Für die Ausführung der Experimente war es notwendig die Konditionen für die FPLC-Gel filtration zu fixieren.
Die Trennung von Actin war allerdings nicht hinreichend. Für eine quantitative Bestimmung mittels Piekoberflächen ist es unbedingt erforderlich, dass das Eiweiss Actin von anderen Muskelproteinen getrennt worden ist.
Eine quantitative Bestimmung für Actin mittels seines 3-MeHis-Gehalt ist vorgeschlagen worden.

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Für dieses Verfahren ist es unbedingt erforderlich, dass das Eiweiss Actin vom Eiweiss Myosin getrennt worden ist und dass die Proteinen die mit Actin ko-eluieren können, frei sind von 3-MeHis Beiträgen. Dieser Bericht beschreibt die analytischen Experimente. Nach der FPLC-Gelfiltration wurde Actin zu 85 (± 10) Prozent nachgewiesen.

Summary

Gel filtration has already been applied for many years to the separation of proteins.

The conventional gel filtration-technique is very time-consuming and limits the number of experiments.

With the introduction of the FPLC it is possible to do the same number of experiments in one day instead of one week.

The FPLC Gel Filtration is applied to the separation of actin out of a mixture of myofibrillar proteins. For these experiments it was necessary to fix the best conditions for FPLC Gel Filtration.

However the separation of actin is not effective enough for its quantification by the peak area in the elution pattern. This fact declares why actin is analysed by its 3-MeHis content. This approach requires, that the actin fraction has to be separated from the myosin fraction, and the proteins which can co-elute with actin, have to be free from 3-MeHis contributions.

This study reports about the analytical experiments. After FPLC Gel Filtration, actin was recovered to a percentage of 85 (± 10).

Abbreviations

DTE = Dithioerythritol

FPLC = Fast Protein Liquid Chromatography

HPLC = High Performance Liquid Chromatography

3-MeHis = 3-Methyl-L-Histidine

MM = Molecular Mass

PAGE = Polyacrylamide Gel Electrophoresis

SDS = Sodiumdodecylsulphate

1. Introduction

In earlier studies [1] on the separation of actin from meat and meat-products it was suggested that the high performance gel filtration-technique versus classical gel filtration should be preferred to reduce time for analysis. With this Fast Protein Liquid Chromatography (FPLC)-technique, also under conditions with effective detergents e.g. sodiumdodecylsulphate (SDS), it is possible to reduce the time of one analysis from 40 h to about 1.5 h.

The question of an effective separation of the muscle protein actin from the natural mixture of myofibrillar proteins will be answered by using the FPLC-technique.

An optimal separation is necessary to quantify actin. If the isolated actin contains residues of other myofibrillar proteins, the follow-up with the determination of 3-Methyl-L-Histidine (3-MeHis) in actin must be examined.

This study describes the experiences with the FPLC-technique for the separation of actin from a mixture composed of some single muscle proteins like myosin, α-actinin, tropomyosin and troponin, and the quantitative analysis of actin.

The effectivity of separation of myosin from actin by the FPLC-technique was simultaneously studied by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). As only low concentrations of protein-bound 3-MeHis are present in the FPLC-fractions, a sensitive method described by Jones et al [2] was used to determine the actin quantitatively.

To assure that 3-MeHis only originated from actin, possible contaminants with a molecular mass close to actin, like α-actinin and tropomyosin, were analysed for this amino acid.

2. Materials and Methods

Scheme 1 illustrates the analytical procedure carried out on the commercial actin preparation.

2.1 Myofibrillar Proteins and 3-MeHis

Commercial protein preparations of actin, tropomyosin, troponin and a suspension of myosin from bovine muscle, and a suspension of α-actinin from chicken gizzard as well as 3-MeHis were obtained from Sigma (USA) with product (-and batch) numbers:
A-3653 (24F-9530), T-4770 (83F-9655), T-4895 (83F-9660), M-6643 (24F-9525), A-9776 (71F-3859) and M-3879 (63F-0313) respectively.

2.1.1 Protein Solution for the FPLC

Actin (6.90 mg) was dissolved in the protein solvent for myofibrillar proteins (7.00 ml) according to De Wreede and Stegemann [3].
The suspension of myosin (1.52 ml containing 13.5 mg protein) was diluted with the protein solvent (to 5.00 ml).
A volume of 2.00 ml of the protein solvent was added to tropomyosin (2.10 mg).
The α-actinin suspension (200 µl; containing 1.92 mg α-actinin) was mixed with 1800 µl of the protein solvent.
The solution process of the proteins in the solvent was completed by stirring the fluids during 2 h at room temperature. Only the myosin solution was filtered through an Acrodisc filter (0.45 micron; Gelman Sciences, UK).
A mixture of commercially available myofibrillar proteins was prepared according to the ratio of the mass composition in myofibrillar tissue

reported by Harrington [4]. This mixture consisted of 320 µl myosin suspension, 10 µl α-actinin suspension, 1270 µl actin solution (5.50 mg/6.00 ml), 300 µl tropomyosin solution (2.10 mg/2.00 ml), 275 µl troponin solution (2.27 mg/ 2.00 ml) and 735 µl De Wreede/ Stegemann [3] solvent.

The mixture was filtered by an 0.45 micron Acrodisc filter. The filtrate was diluted with solvent in a ratio of 1:1.

2.2 FPLC Gel Filtration

SDS-gel filtration of the proteins was carried out with a Fast Protein Liquid Chromatography system of Pharmacia (Sweden), consisted of: one P-500 pump, a liquid chromatography controller LCC-500, two electrical powered motor valves (MV-7 and MV-8), a single path monitor UV-1, a peristaltic pump, a FRAC-100 fraction collector, and a recorder. To study the experimental conditions the calibration proteins were separated on two TSK G4000SW columns of different lenghts (30 and 60 cm; inner diameter of 7.5 mm) obtained from Chrompack (the Netherlands). The upper exclusion limit of this type of column is 4 x 10^5 Daltons.

A gel filtration calibration kit (no. 17-0442-01; lot no. 4087) of Pharmacia (Sweden) with Albumin (67,000), Ovalbumin (43,000), Chymotrypsinogen A (25,000) and Ribonuclease A (13,700) was used.

Optimum conditions for separation of these calibration proteins were investigated by subsequently varying the flow rate (from 6.00 ml/h to 90.00 ml/h), phosphate concentration of the elution buffer (from 0.200 to 0.001 mol/l), the percentage of SDS in the solution (from 0.05 to 2.00%), the pH (from 3.5 to 7.5 by steps of one pH unit) and the column length (30, 60 and 90 cm).

After the fixation of optimum conditions the proteins were eluted by a flow rate of 30 ml/h with a buffer solution of $NaH_2PO_4,1H_2O$ (0.014 mol/l) and $Na_2HPO_4,2H_2O$ (0.036 mol/l) with a pH of 6.5, containing 0.10% (m/v) SDS. One run took about 1 h 30 min. The absorbance of the eluted proteins was measured at a wavelength of 280 nm, followed by registration of the elution pattern. The protein fractions of the main peaks in the elution pattern were separately gathered by a fraction collector and stored for further analysis.

2.3 SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was applied according to the modified procedure of Hofmann and Penny [5] as described by Van Bergen and Van den Bosch [6] in a Protein Dual 16 cm Vertical Slab Gel Electrophoresis Cell (Bio Rad, USA). The protein pattern in the electropherogram was calibrated by the standard proteins Phosphorylase b (94,000), Albumin (67,000), Ovalbumin (43,000), Carbonic Anhydrase (30,000), Trypsin Inhibitor (20,100) and α-Lactalbumin (14,400) (Kit No. 17-0446-01 lot no. 5113; Pharmacia, Sweden). For the electrophoresis of the actin- and myosin-containing fractions of the FPLC, 40 µl volumes were used out of total volumes of respectively 5 and 7 ml.

2.4 3-MeHis Determination

2.4.1 Hydrolysis

2.4.1.1 Hydrolysis of Proteins present in Eluted Fractions

A volume of 4.00 ml of each actin-containing FPLC fraction (5.00 ml was collected) as well as volumes of 500 µl of the original actin solution and each of the other myofibrillar protein solutions (made up to 4.00 ml with deionized water) were mixed with 4.00 ml of 37% (m/m) HCl. The hydrolysis took 22 h under boiling conditions with reflux. After this step, samples were made up with 6 mol/l HCl to a volume of 10.00 ml in a calibration vessel. The hydrolysates were stored at 4°C

until the procedure was continued. Each hydrolysate (2.00 ml) was neutralised with 8 mol/l NaOH (1.50 ml) and directly used for derivatisation of 3-MeHis.

2.4.1.2 Hydrolysis of Tropomyosin and α-Actinin

Tropomyosin (0.53 mg) and α-Actinin (50 µl of the original suspension) were dissolved separately in protein extraction fluid up to a volume of 500 µl. After addition of 3.5 ml deionized water to each of the solutions the above mentioned procedure for hydrolysis was followed.

2.4.2 Derivatisation of 3-MeHis

The procedure for imidazole amino acid derivatives of Jones et al [2] was followed. Because of the low concentrations 3-MeHis present in the FPLC Gel Filtration fractions the usual volumes of this method were changed. Each hydrolysed and neutralised sample solution (1.00 ml) was mixed with 0.2 mol/l $Na_2B_4O_7$,10 H_2O (2.50 ml) of pH 9.0.
The derivatisation solution (2.00 ml) of fluorescamine (2.50 mg/ml acetonitril) from Pierce (USA) was added to the sample/borate buffer mixture. After vigorously shaking 2.00 ml of 2.5 mol/l HCl was added. Standard 3-MeHis solutions of 25, 50 and 75 ng/ml were prepared by dilution from a stock 3-MeHis solution containing 500 µg 3-MeHis per ml. The standard solutions were treated in the same way as the samples.

2.4.3 HPLC of the 3-MeHis-Fluorescamine Component

High Performance Liquid Chromatography (HPLC) of 3-MeHis-fluorescamine was applied with a Chromspher C_{18} cartridge column (length and inner diameter resp. 100 mm and 3 mm) from Chrompack (the Netherlands). The HPLC apparatus (Waters Associates, USA) consisted of the system controller (model 720), sample processor (WISP 710 B), solvent deli-

very system (model 6000 A), temperature control module and column heater, fluorescence detector (model 420) and data module (model 730). The fluorescence detector was equipped with an 'aflatoxin' lamp (nr. 78245; Waters Associates, USA) an excitation filter near 360 nm and an emissionfilter for wavelengths above 420 nm.

Volumes of 100 µl of the 3-MeHis derivatives of samples and standard solutions were injected onto the cartridge column.

The isocratic elution was effectuated at a flow rate of 30 ml/h using a buffer solution of 40% (v/v) methanol and 60% (v/v) sodium acetate (1.0 g sodium acetate and 2.5 g acetic acid in 1 l deionized water, adjusted to a pH of 4.0).

3. Results and Discussion

3.1 Fast Protein Liquid Chromatography by Gel Filtration

The experimental conditions and experiences of the classical gel filtration chromatography [7] were used to standardize fast gel filtration chromatography of proteins. The initial system consisted of a mixture of standard proteins (Albumin, Ovalbumin, Chymotrypsinogen A and Ribonuclease A). The proteins were eluted at a flow rate of 6.00 ml/h in a buffer solution (pH 6.5) of 0.10 mol/l phosphate and 0.20% (m/v) SDS on the TSK G4000SW column with a length of 30 cm. The elution pattern of the mixture of standard proteins at different conditions of flow rate, phosphate concentration, percentage of dodecylsulphate, pH and columnlength were judged by peak resolution (Table 1). Generally, the differences in peak resolution were small. Nevertheless, the best choices were made.

At lower flow rates the peak resolution is improved but does not result in completely separated proteins. However, decreasing flow rates cause

increasing elution times. As a compromise a flow rate of 30.00 ml/h was chosen. The best results were obtained at a concentration of 0.050 and 0.010 mol/l phosphate buffer solution. Below 0.010 mol/l phosphate the standard proteins elute much faster, resulting in a poorer resolution. The exchange of the proteins between 'the stationary' (liquid inside the particles) and the mobile phase of the column, decreases with lower concentrations of phosphate in the solutions [8]. However, a greater buffer capacity was preferred considering the pH stability in future applications. A concentration of 0.050 mol/l phosphate was chosen. Going down from 2.00 to 0.05% sodiumdodecylsulphate, some improvement of peak resolution was observed. This improvement disappeared below 0.10% SDS. The different pH values of the elution fluid yielded an optimum peak resolution of the elution patterns at a pH of 6.5.

The separation of the proteins depends on the number of theoretical plates of the column. This number was increased by use of a longer column (90 cm instead of 30 cm) with the same inner diameter. Peak resolution in the elution pattern was strongly improved with a column length of 90 cm. A longer column cannot be used, because the column backpressure will increase above the maximum admissible level (2.5 MPa). Table 2 shows the elution volumes of the different standard proteins in relation to the chosen lengths of the column; the peak resolution increases with a longer column. The elution volumes of the standard proteins for a column lenght of 90 cm were not used for calibration in further experiments because the choices of optimum separation conditions were introduced afterwards.

3.1.1 Localisation of Actin and other Myofibrillar Proteins in the Elution Pattern

Adsorption of SDS to protein molecules increases the molecular mass of these proteins by about 2.4 times [9]. The molecular mass has to be within the exclusion limits of the applied gel. The TSK G4000SW column has the highest separation efficiency at a molecular mass of more than 60,000 D. Using a 90 cm TSK-column, the following linear relationship between the K_{av} value and the log MM of the standard proteins was calibrated again:

$$K_{av} = 2.61 - 0.47 \log MM \qquad (I)$$

in which K_{av} is defined as (Ve-Vo)/(Vt-Vo). The K_{av} gives the available portion of the intra-particle volume (Vt-Vo) to a protein molecule. The void volume V_o and the end volume V_t are equal to 15.3 ml and 39.0 ml respectively. The elution volume V_e depends on the size of the eluted protein.

The FPLC elution pattern of a solution of commercially available actin (0.99 mg/ml) in Fig. 1 shows an elution volume V_e of 25.4 ml for actin. This preparation gives a clear peak of the main component actin. The high peak at the end of the run corresponds to DTE from the protein extraction fluid. Close to the actin peak very small contributions in absorbance by contaminants are observed. These contaminants in the commercial actin preparation originate probably from other myofibrillar proteins.

The elution volumes of myosin, α-actinin, actin, tropomyosin and troponin were determined by separate FPLC-runs of these myofibrillar proteins. After gel filtration of the mixture the proteins myosin, α-actinin, actin and troponin were eluted as separate peaks; tropomyosin appeared as a shoulder of the actin peak (Fig. 2).

The molecular masses of the myofibrillar proteins, except myosin, were calculated from the elution volumes by equation I and were compared (Table 3) with values from literature [4]. (Myosin appears outside the exclusion range of the column). These values agree with the localisation of the separate proteins in Fig. 2.

The impurities in the actin preparation are identified as α-actinin and troponin. Fig. 2 shows a very good separation between the proteins myosin and actin. Actin and troponin were separated effectively. However, a less effective separation is observed in the molecular mass area of actin. Presence of α-actinin and tropomyosin in the actin-containing fraction has to be expected, which may cause interference with the quantitative determination of actin from peak area measurements. Because of the specific content of 3-MeHis in actin, one mol/mol actin [10], it should be possible to quantify the actin content in the fraction by means of a 3-MeHis determination. In that case it is essential that actin has been completely separated from the other 3-MeHis-containing myofibrillar protein myosin, ranging from zero, one or two mol/mol myosin [11].

3.2 SDS-Polyacrylamide Gel Electrophoresis

After FPLC Gel Filtration of actin and myosin solutions (Fig. 3) the actin-containing fraction (D) and the myosin-containing fraction (A) and the two intermediate fractions (B and C) were applied for electrophoresis. The results from Fig. 3 show a complete absence of actin and myosin in the intermediate fractions. Actin shows two separate protein-components in the pherogram which were already observed [7].

3.3 Analysis of 3-MeHis

3.3.1 3-MeHis Determination in Actin

The main purpose of this study is the comparison of the quantities of 3-MeHis in actin before and after the FPLC.

Fig. 4 shows the HPLC chromatogram of 3-MeHis analysis in the original actin preparation (I), the elution pattern of actin after FPLC (obtained by the FPLC printer) and the HPLC chromatogram of 3-MeHis analysis in the actin-containing fraction (II).

The HPLC chromatograms have similar amino acid patterns for 3-MeHis and histidine (His). The peak areas of 3-MeHis in the HPLC chromatograms of the actin preparation and the actin-containing fraction are given in Table 4. As 80% of the volume of the collected actin fraction was applied for 3-MeHis determination, the 3-MeHis peak area was multiplied by a factor of 1.25, to get the values mentioned for the actin-containing fractions in Table 4. The values for 3-MeHis show that after FPLC an average percentage of 85 (± 10) of the original amount of 3-MeHis was detected. The calculation of the amount of actin (Table 5), based upon 1 mol 3-MeHis per mol actin, shows that the actin preparation has an actin content of 71%. The impurities in the actin preparation were already shown in Fig. 1.

3.3.2 3-MeHis Determination in other Myofibrillar Proteins

The actin separated by the FPLC method is not contaminated by myosin (fig. 3). Other myofibrillar proteins however can co-elute with actin. It is important to know that these proteins do not contribute to the 3-MeHis content.

In this case α-actinin and tropomyosin are the more possible contaminants (Fig. 2). Fig. 5 shows the chromatograms of the analyses of 3-MeHis in concentrated [2.4.1.2] solutions of α-actinin and tropomyosin. No 3-MeHis was detected in the solution of both proteins.

4. Conclusions

The FPLC Gel Filtration-technique is a rather adequate technique to separate actin from other myofibrillar proteins.
This technique can be applied with effective detergents as SDS, which is important for future research.
The time of analysis is very short compared to the conventional method.
However, also under optimum conditions a complete separation of actin from proteins in the molecular mass range of 30,000 to 100,000 was not possible in the applied system.
A quantitative method of determination of actin can be based on its 3-MeHis content, after separation of this protein from myosin by FPLC.

Acknowledgement

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Legend to the figures

Fig. 1. The FPLC Gel Filtration pattern of a solution (500 µl) of commercial actin (6.90 mg/7.00 ml solvent).

Fig. 2. The FPLC Gel Filtration pattern of a mixture (100 µl) of actin and other important myofibrillar proteins. The ratio of proteins in the mixture agrees with 59% myosin, 2% α-actinin, 25% actin, 7% tropomyosin and 7% troponin.

Fig. 3. The FPLC Gel Filtration pattern of 500 µl actin solution (5.50 mg/6.00 ml solvent) and 500 µl myosin solution (mixture of 1.52 ml myosin suspension and 3.48 ml solvent). Schematic presentation of SDS-PAGE of the eluted fraction with myosin, intermediate fractions and fraction with actin.

Fig. 4. Determination of 3-MeHis in actin in two ways.

I. Only by HPLC after acid destruction of 500 µl actin solution (0.99 mg/ml solvent).

II. Gel filtration of the same quantity of the actin solution by FPLC and procedure I.

Fig. 5. Chromatograms of the analysis of 3-MeHis in concentrated solutions of α-actinin and tropomyosin.

Scheme I

Scheme of analysis of the commercial actin preparation

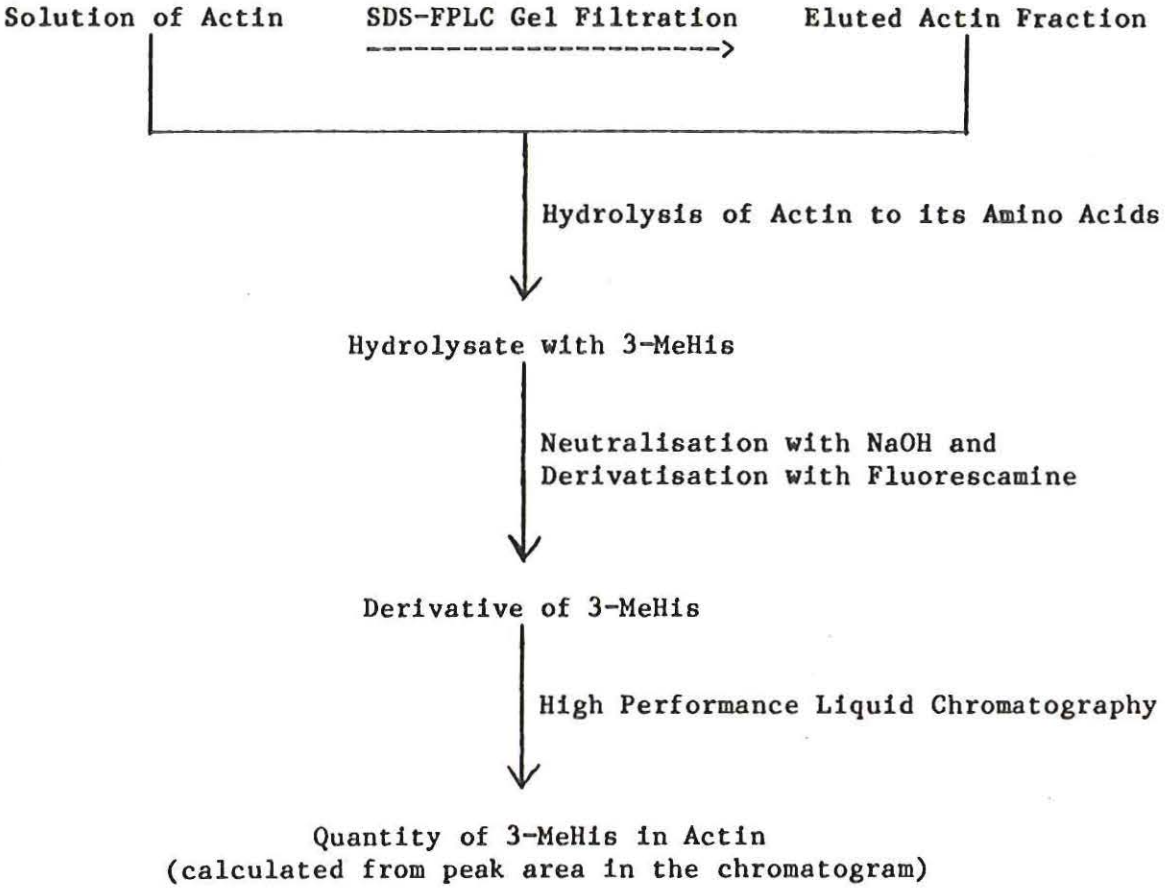


Table 1
Judgement of peak resolution by varying the conditions of the FPLC Gel Filtration of standard proteins

Flow rate		Phosphate		SDS		pH		Column length	
ml/h	Peak*) reso-lution	mol/l	Peak*) reso-lution	% (m/v)	Peak*) reso-lution	Value	Peak*) reso-lution	cm	Peak*) resolu-tion
						3.5	-		
6.00	+	0.001	-			4.5	-	30	-
15.00	+-	0.010	+-	0.05	-	5.5	-	60	+-
30.00	+-	0.050	+-	0.10	+-	6.5	+-	90	+
60.00	-	0.100	-	0.20	-	7.5	-		
90.00	-	0.200	-	0.50	-				
				2.00	-				

*) + = sufficient
+- = moderate
- = insufficient

Table 2
The elution volumes (in ml) of standard proteins by different lengths (in cm) of the TSK G4000SW column

Standard Proteins	Mol Mass	Elution Volume by a column lenght of		
		30	60	90
Albumin	67,000	7.78	15.73	23.39
Ovalbumin	43,000	8.24	16.78	25.01
Chymotrypsinogen A	25,000	8.82	17.99	26.64
Ribonuclease A	13,700	9.64	19.82	29.16
V_o		4.95	10.12	14.92
V_t		13.19	25.80	38.00

Table 3
Protein identification in the elution pattern (Fig. 2) by comparing calculated mol mass values (in Daltons) and mol mass values from literature [4]

Proteins	V_e*) (ml)	K_{av}	MM**) (found)	MM (from literature)
α-Actinin	22.6	0.31	86,000	90,000
Actin	25.4	0.43	47,000	42,000
Tropomyosin	27.4	0.51	32,000	35,000
Troponin	30.6	0.65	16,000	18,000
-	35.0	0.83	6,600	

*) = data from Fig. 2
**) = calculated with equation I

Table 4
Measured peak areas (in mm^2) of 3-MeHis in the HPLC chromatograms for actin and actin-containing fractions, and their average 3-MeHis contents (in ng)

Sample	n*)	Area		3-MeHis**)
		Found	Mean	
Actin	4	169 151 161 180	165	1.07
Actin-containing fraction	5	135 141 133 160 138	141	0.91

*) = number of determinations
**) = ng 3-MeHis/100 µl injection volume in HPLC

Table 5
Quantity of 3-MeHis (in ng) and actin (in mg) found in the commercial actin solution and the corresponding actin-containing fraction of the FPLC Gel Filtration

Sample	Sample Volume (in ml)		Absolute Quantity	
	before FPLC	after FPLC	3-MeHis found	Actin found
Actin preparation (0.99 mg/ml)	0.500		88	0.35
Actin-containing fraction from FPLC		5.00	75	0.30

Fig. 1

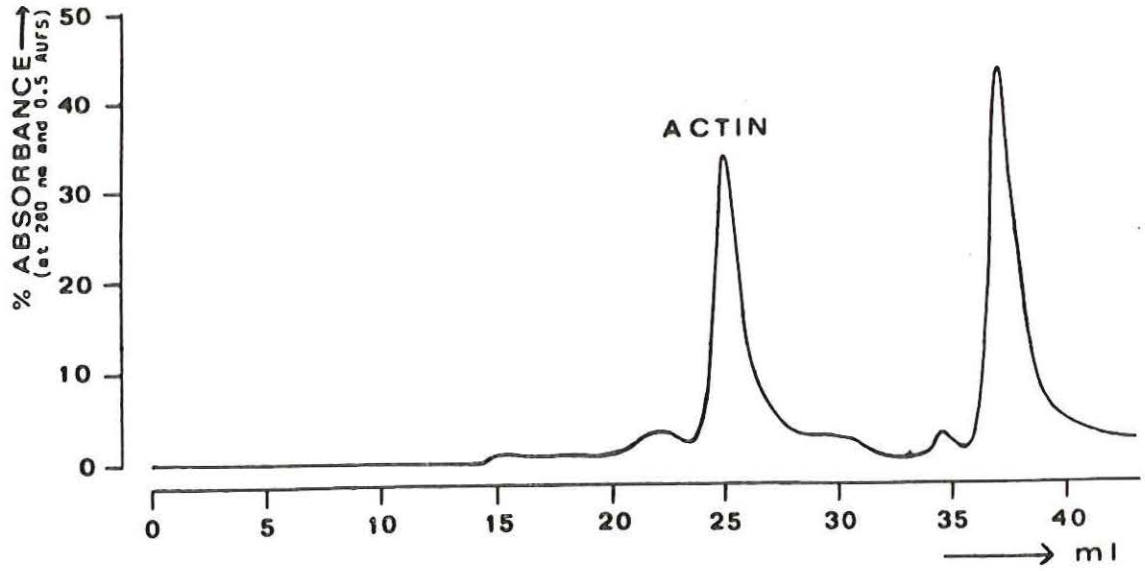

% ABSORBANCE →
(at 280 nm and 0.5 AUFS)
50
40
30
20
10
0
ACTIN
0
5
10
15
20
25
30
35
40
→ ml

Fig. 2

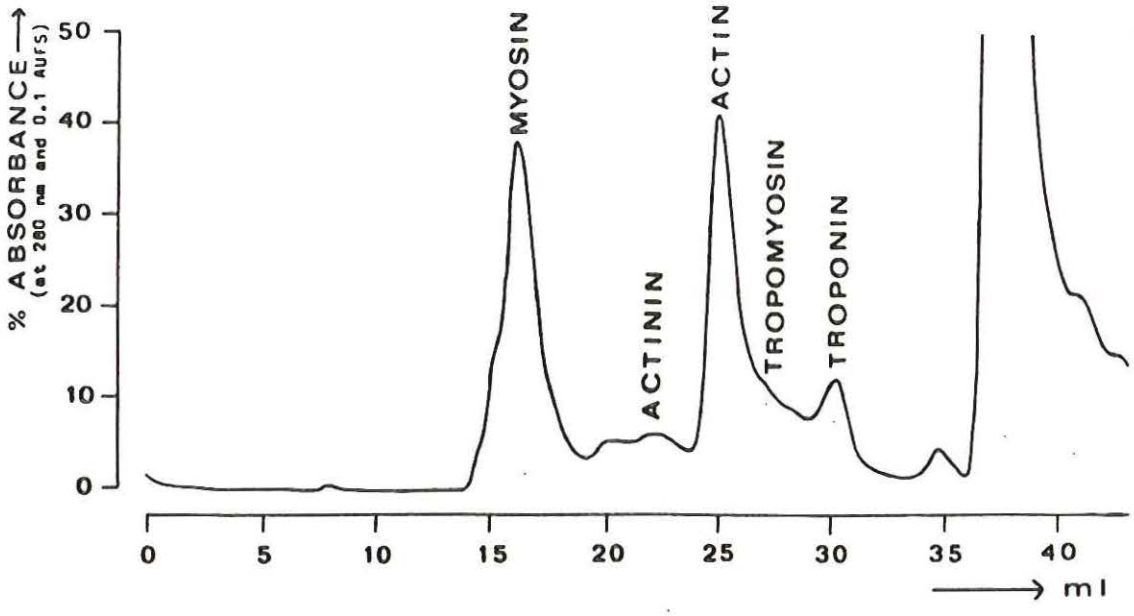
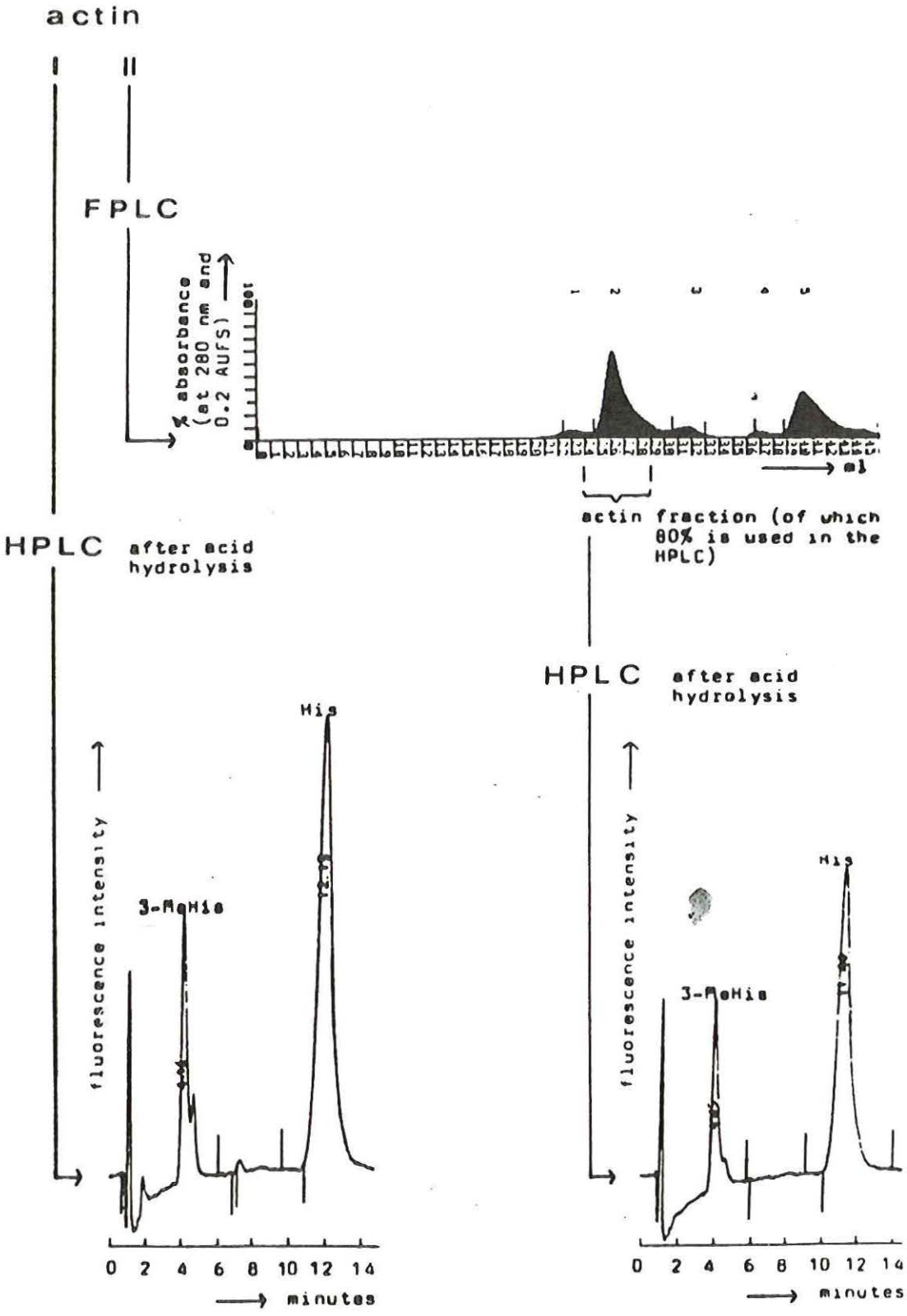

% ABSORBANCE →
(at 280 nm and 0.1 AUFS)
50
40
30
20
10
0
MYOSIN
ACTININ
ACTIN
TROPOMYOSIN
TROPONIN
0
5
10
15
20
25
30
35
40
→ ml

Fig. 4


actin
I
II
FPLC
% absorbance (at 280 nm and 0.2 AUFS)
ml
actin fraction (of which 80% is used in the HPLC)
HPLC after acid hydrolysis
fluorescence intensity
3-MeHis
His
0 2 4 6 8 10 12 14
minutes
HPLC after acid hydrolysis
fluorescence intensity
3-MeHis
His
0 2 4 6 8 10 12 14
minutes

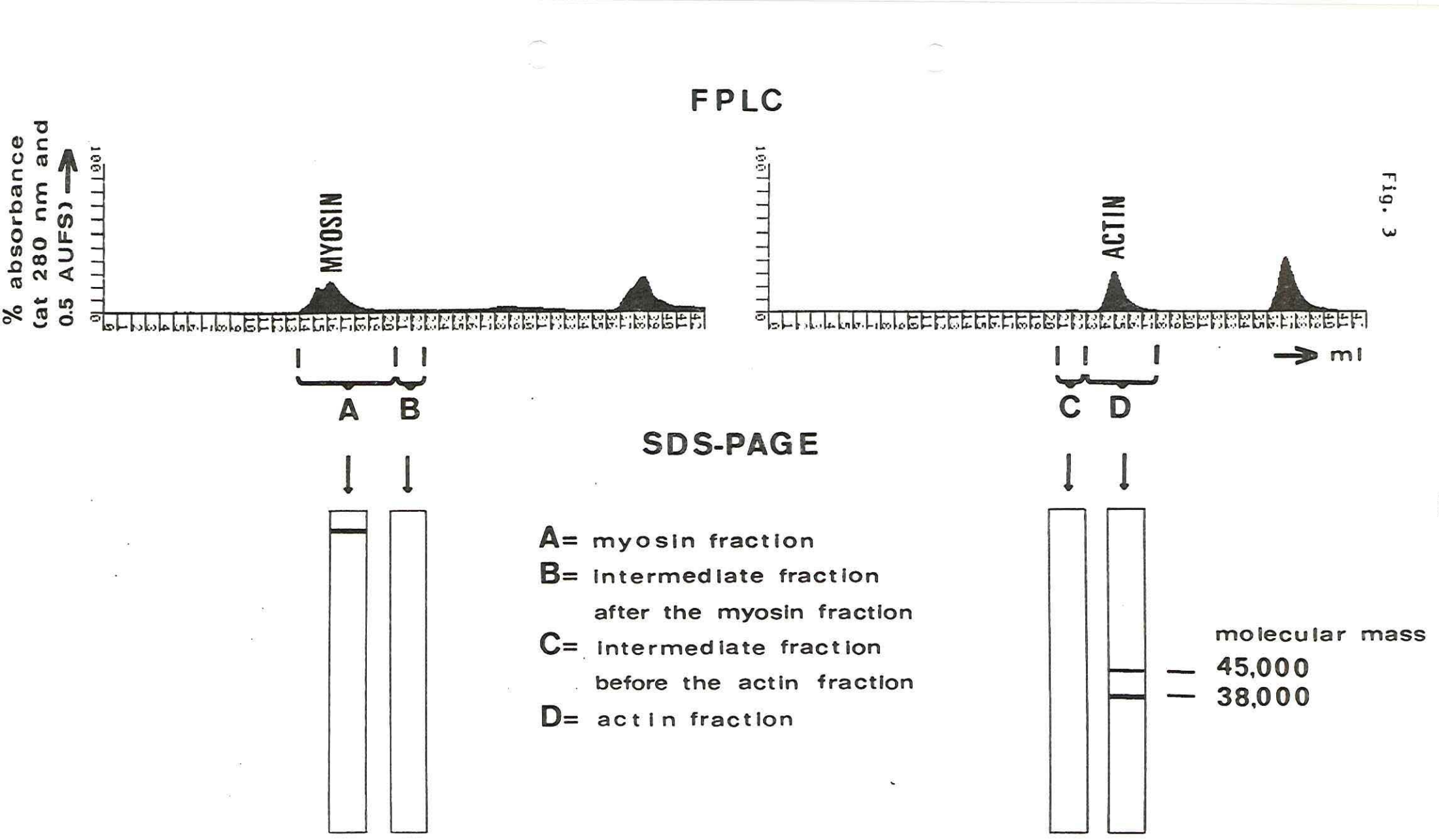
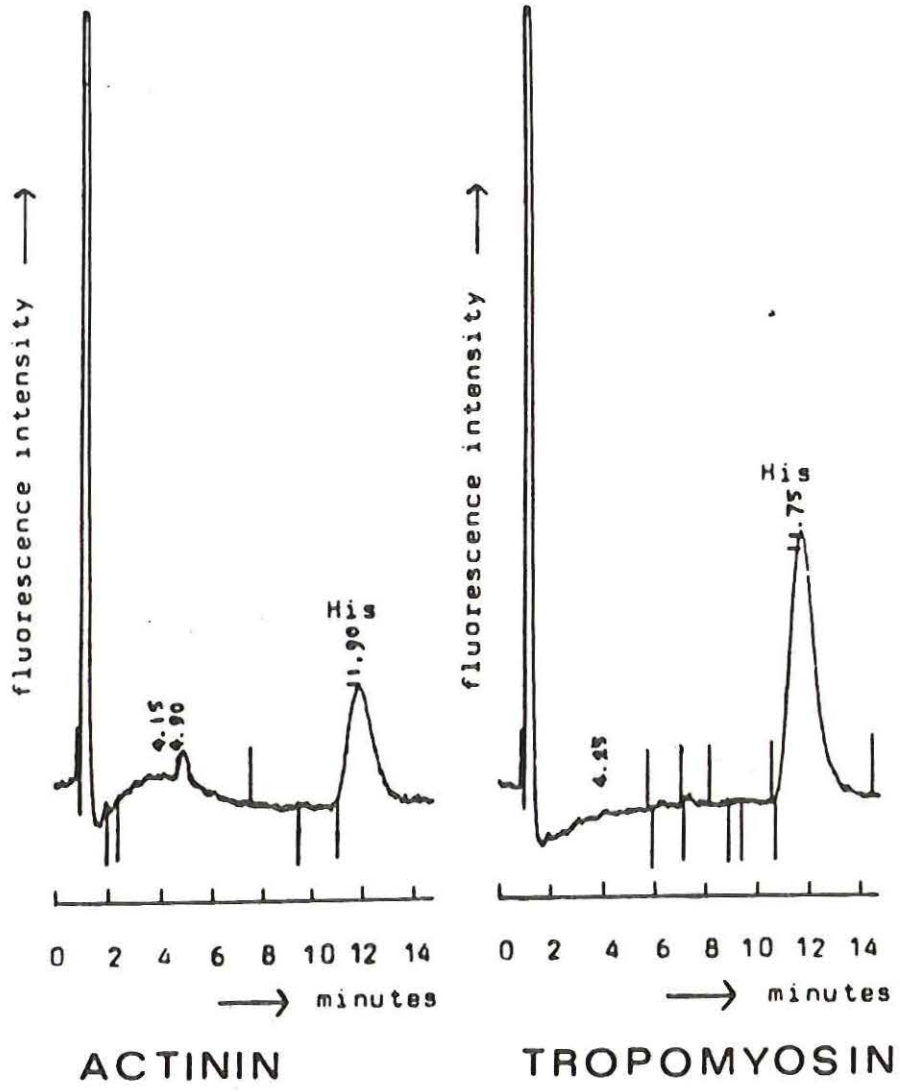

FPLC
% absorbance (at 280 nm and 0.5 AUFS)
MYOSIN
ACTIN
ml
A
B
C
D
SDS-PAGE
A= myosin fraction
B= intermediate fraction after the myosin fraction
C= intermediate fraction before the actin fraction
D= actin fraction
molecular mass
45,000
38,000

Fig. 3

Fig. 5


fluorescence intensity
His
11.90
9.15
8.90
0 2 4 6 8 10 12 14
minutes
ACTININ
fluorescence intensity
His
11.75
4.25
0 2 4 6 8 10 12 14
minutes
TROPOMYOSIN