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IDENTIFICATION OF ACTIN ISOFORMS AFTER *IN SITU* HYDROXYLAMINE CLEAVAGE ON SEQUENCER MEMBRANES: SERUM ACTIN IS A CYTOPLASMIC ISOFORM

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Summary: Actin was isolated from fetal bovine serum by means of affinity chromatography on DNase I-Sepharose, reversed phase chromatography and SDS-gel electrophoresis. It was identified as a cytoplasmic isoform by direct micro sequence analysis of hydroxylamine cleavage products which had been generated *in situ* after covalent linkage of the protein to sequencer membrane disks through arylamine or isothiocyanato functionalities.

Actin is a major component of eukaryotic cells with mainly contractile and structural functions. Its quaternary structure, and thereby these functions, is modulated by many proteins (1). In addition its ability to interact with a large number of proteins has lead to proposals that actin may have yet other functions in vivo (2-4). Support for this hypothesis is provided by the fact that actin is found not only inside the cells, but also on the cell surface (5,6), and in the extracellular matrix (7), serum (8), and other biological fluids (9). Actin is highly conserved in evolution, and isoforms have been described in many cells and tissues. These usually fall into two classes: muscle and non-muscle (or cytoplasmic) actins. Minor differences within each class are localized mainly in the N-terminal region of the molecule (10). A third, much different, actin class has recently been identified in vertebrates (centractin, (11-13)), and a fourth vertebrate class is formed by bovine actin2 (14). This multiplicity of actins may have physiological significance since intra- and extracellular localization and some biochemical reactions are specific for different actin isoforms (reviewed in ref. 11,15,16). For example, muscle actin is preferentially selected for myofibril assembly while cytoplasmic actin usually forms the cytoskeleton. Cytoplasmic actin binds the capping protein profilin and undergoes *in vitro* ADP-ribosylation much more effectively than muscle actin.

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Abbreviations: His(3Me), 3-methyl-histidine; PAGE, polyacrylamide gelelectrophoresis; SDS, sodium dodecylsulphate.

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The two major actin classes have >90% sequence identity, so that clear identification of actin isoforms in any given case is rather difficult. They are usually distinguished with the help of partially specific antibodies or by electrofocussing, whereas more convincing approaches would rely on the analysis of primary structure. Direct sequencing of the actin molecule is not feasible since its N-terminus is acetylated. Isolation of the amino terminal peptide is frequently employed (17), but other experimental approaches are needed in instances where only small amounts of actin are available, and especially since actin is notoriously difficult to purify at such levels. We have developed a novel procedure for identifying actin isoforms. It is based on the presence of a unique Asn_{12}-Gly_{13} sequence in all of the vertebrate actin species (14,15)(except for bovine actin2, which has no Asn-Gly sequence), which precedes the sequence that distinguishes four subsets of these isoforms (Leu_{16}-Val, Leu-Cys, Met-Cys, and Val-Ile, for α-skeletal/cardiac, smooth muscle and cytoplasmic isoforms, and centractin, respectively), which has afforded an appropriate tactic. Asn-Gly sequences can be cleaved specifically by hydroxylamine (18). Since the N-terminal of actin is blocked, direct sequencing of an unfractionated digest should therefore give only one major, characteristic sequence. Hydroxylamine cleavage of small amounts of protein *in situ* in polyacrylamide gels is feasible (19) but it has not been possible to sequence the resultant peptides. We have exploited the availability of sequencer membrane disks with isothiocyanato or arylamine functionalities (20) to covalently couple actin to such a disk, digest with hydroxylamine, wash away the salts and reagents, and directly sequence the fragments. This procedure allows sequencing of very small amounts of protein, including protein samples extracted from electrophoretic gels (21).

MATERIALS AND METHODS

Fetal bovine serum was from HyClone and Gibco , DNase I from Boehringer and bovine skeletal muscle actin from Sigma. DNase I-Sepharose was prepared from CNBr-Sepharose (Pharmacia) according to the manufacturer's procedure (24.5 mg DNase-I to 5 g of CNBr-Sepharose). $CaCl_2$ (1mM) was included in the coupling buffer. Recombinant angiogenin (M30L), with amino terminal Gln, was provided by Hoechst, A.G. (Frankfurt).

Isolation of actin from fetal bovine serum: Affinity chromatography of serum on DNase I-Sepharose (2) was accomplished at a ratio of 10 ml serum to 1 ml of swollen gel at 4° C. Serum was adjusted to 10 mM Tris (pH 7.5) and 5 mM $CaCl_2$, and applied to the column at 0.5 ml/hr. The column was thoroughly washed with standard buffer (10 mM Tris, 5 mM $CaCl_2$, pH 7.5) followed by 0.75 M guanidine hydrochloride, then 0.5 M NaCl (all solutions in standard buffer) and finally the bound material was eluted at room temperature with 3% SDS in water. Eluted material was adjusted to 0.1% trifluoroacetic acid and passed through a C4 column (Supelguard LC-304, Supelco) at 1 ml/min (4). Elution was carried out with a linear acetonitrile gradient 0 - 75% in 0.1% TFA for 40 min. Fractions of 0.5 ml were collected. Electrophoresis was done according to Laemmli in a 10% gel (22). After electrophoresis the gel was stained for 5 min in 0.05% Coomassie R-250 solution in 50% methanol, 7% acetic acid, rinsed twice with water and the p43 band was cut out. The gel slice was washed 3 times with 0.5 ml water (5-10 min each) and extracted overnight at 37° C with 0.1 M sodium acetate, 0.1%

SDS, pH 8.2, with shaking (21). Control experiments demonstrated that more extensive wash of gel slices before extraction leads to considerable losses of actin.

Hydroxylamine cleavage and sequencing: Protein solutions were dried onto isothiocyanato or arylamine membranes (Sequelon DITC and -AA, Millipore) and coupling to the membranes followed the manufacturer's suggestions. Cleavage buffer was 2 M hydroxylamine hydrochloride, adjusted to pH 9 with solid KOH, and then made 0.2 M in potassium carbonate. The protein-membranes were incubated in 1 ml of cleavage buffer for 16 hours at room temperature (23 °C), rinsed briefly with water (2x) and methanol (2x) and installed in a sequencer cartridge (ProSequencer, Millipore). A Millipore ProSequencer, using the Cov-100 program was employed for sequencing; 100% of each cycle was injected for HPLC identification of the phenylthiohydantoin amino acids.

Amino acid analyses: 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) methodology (Waters AccQ.Tag, Millipore)(23) was used. The methylhistidine derivatives elute as completely resolved peaks between those of ammonia and arginine. AQC-His(3Me) elutes 0.9 min before AQC-Arg and 1.0 min after AQC-His(1Me) and is quantifiable at sub-picomol levels.

RESULTS

Sequencing of standards, cleaved with hydroxylamine: The hydroxylamine cleavage/sequencing methodology was established on two known proteins. Angiogenin (6 μg, 450 pmol)(24), linked covalently to a DITC membrane and cleaved with hydroxylamine, was sequenced and yielded two major and one minor simultaneous sequences. These were the amino terminal sequence (indicating a coupling yield of 75%), the sequence following from cleavage at Asn_{61}-Gly (hydroxylamine cleavage yield of 23%), as well as that following from cleavage at Asn_3-Ser (cleavage yield of 6%). Table 1 summarizes these data. When arylamine membranes were used, the sequencing yields of angiogenin and the cleavage sequences were a third of these, but signalling much the same efficiency of hydroxylamine cleavage as in the DITC experiment. In addition, a second Asn_{110}-Gly sequence could be followed in a peptide which was linked

Table 1. Sequencer results for the hydroxylamine digest of angiogenin

Cycle #	Yields of PTH-amino acids (pmol)	Expected Sequences		
		1	62	4
1	Gln (26), Gly (125), Ser (7)	Gln	Gly	Ser
2	Asp (82), Asn (76), Arg (20)	Asp	Asn	Arg
3	Asn (48), Pro (40), Tyr (22)	Asn	Pro	Tyr
4	Ser (+), His (33), Thr (12)	Ser	His	Thr
5	Arg (73), His (30)	Arg	Arg	His
6	Tyr (40), Glu (49), Phe (22)	Tyr	Glu	Phe
7	Thr (25), Asn (36), Leu (17)	Thr	Asn	Leu
8	His (32), Leu (41), Thr (+)	His	Leu	Thr

450 pmol of a protein preparation with 25% Gln and 75% pyroglutamine as amino terminus (as determined by sequence analysis using a spinning cup sequencer) was attached covalently to a DITC membrane, cleaved with hydroxylamine, and sequenced in a Milligen Prosequencer.

Table 2. Sequencer results on muscle and serum actin after hydroxylamine cleavage *in situ* on sequencer membranes

Sequencer Cycle #	PTH-amino acids (pmol)		Expected sequence	
	Muscle actin	Serum actin	Muscle actin	Cytoplasmic actin
1	Gly (60)	Gly (36)	Gly	Gly
2	Ser (5)	Ser (3)	Ser	Ser
3	Gly (34)	Gly (22)	Gly	Gly
4	Leu (19)	Met (14)	Leu	Met
5	Val (17)	δAla (+)	Val	Cys
6		Lys (3)	Lys	Lys
7		Ala (9)	Ala	Ala
8		Gly (19)	Gly	Gly
9		Phe (9)	Phe	Phe
10		Ala (10)	Ala	Ala

covalently to the arylamine membrane, but which was washed out of the DITC membrane. Bovine skeletal muscle actin (10 μg) bound to a DITC membrane yielded one major sequence, due to cleavage at Asn_{12}-Gly, in 10% overall yield and clearly identified this as a muscle type actin (Leu-Val at cycles 4 and 5) (Table 2).

Purification of actin from fetal bovine serum: Fetal bovine serum (12 ml) was applied to a 1.2 ml column of DNase I-Sepharose as described in "Materials and Methods". The SDS-PAGE electrophoretic pattern of the material that eluted with 2.5 ml of 3% SDS showed five major bands with approximate molecular masses of 43, 58, 65, 78 and 93 kDa.

The volume of 3% SDS needed for effective elution of the material bound to the affinity column usually exceeded two column volumes and therefore created technical difficulties for subsequent procedures. Routine methods of concentration are not applicable in this case due to the high concentration of SDS in the sample. Therefore it was necessary to use reversed phase chromatography on a C4 column to remove SDS and, simultaneously, some protein contaminants. A separation profile of the SDS-eluted material on a C4 column is shown in Fig. 1. SDS has a low affinity for the C4 column and emerges in the flow-through fraction and in the first third of the gradient. Aliquots (50 μl) of each chromatographic fraction were dried in a SpeedVac centrifuge, the residues were dissolved in sample buffer (22) and analysed by PAGE (Fig. 1, inset).

Appropriate fractions containing p43 were pooled, dried in a SpeedVac centrifuge, dissolved in sample buffer and fractionated by PAGE. The protein bands were visualized by staining with Coomassie Blue and the p43 band was eluted from the gel as described in "Materials and Methods". The amino acid composition of the extracted material demonstrated marked similarity to actin even without subtracting background amino acids that arise from the

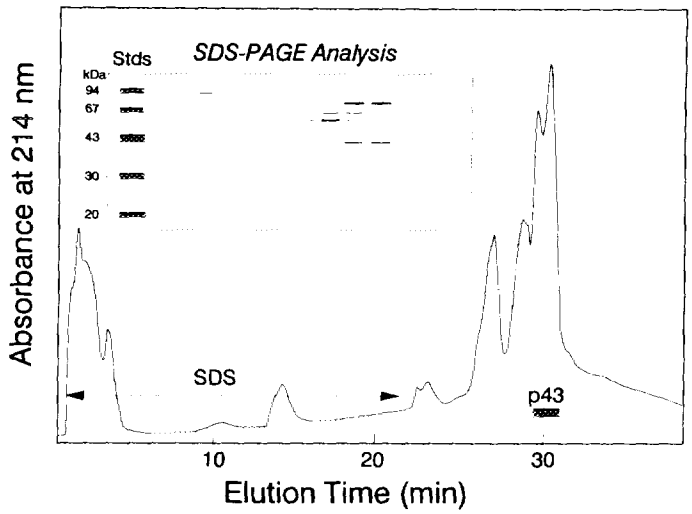

Figure 1. Fractionation of serum components bound to and eluted from DNase I-Sepharose. The 3% SDS-eluate from the DNase affinity column was loaded on a Supelguard C4 column in 0.1% TFA and eluted with a 40 min gradient to 75% acetonitrile.
Inset: PAGE-analysis of column fractions. Aliquots (50 μl) of the indicated fractions were dried in a SpeedVac centrifuge, dissolved in sample buffer and electrophoresed. The gel was stained with Coomassie R-250. The standards were from Pharmacia.

gel. The presence of methylhistidine (His(3Me)) (0.65 residue/mol) in p43 gave additional and very strong evidence that this protein is actin since this rare residue is very characteristic for actin (25). Based on the His(3Me) content, the total amount of actin obtained from 12 ml of fetal bovine serum was about 100 pmol or 4 μg. The real content of this protein in serum should be much higher, since considerable losses of actin take place during purification.

Sequencing of fetal bovine serum actin after hydroxylamine cleavage: Table 2 summarizes the sequence study on the hydroxylamine digest of actin isolated from fetal bovine serum. About 4 μg of serum actin was applied to the DITC membrane and a sequencing yield of 10-15% was attained. The sequence clearly contains Met_{16} and is consistent with Cys_{17}, as indicated by the presence of PTH-dehydroalanine, a characteristic product formed on sequencing through a Cys residue.

DISCUSSION

Hydroxylamine cleavage of proteins is fairly specific for Asn-Gly sequences, although other sequences involving Asn-X linkages are also susceptible to cleavage but at a much lower rate (18). The sequencing yields of 10-17% are only moderate and reflect inefficiency in coupling to the arylamine and DITC membranes (20), as well as incomplete cleavage. Nevertheless, cleavage was relatively specific and enabled clear identification of the sequences following the cleavage points.

Mejean et al. (26) used an enzyme-linked immunosorbent assay and demonstrated that about 99% of the total actin-like immunoreactive material in human plasma is not recognized by antibodies against skeletal actin. One could infer that serum actin is of the cytoplasmic type. The present method has shown directly that actin present in fetal bovine serum belongs to the cytoplasmic type, although it does not discriminate between the highly similar β- and γ-isoforms.

A clear function or origin for circulating actin is not yet known, although embryonic muscle cells, *in vitro*, are found to selectively shed cytoplasmic actins into the medium (27,28). The isoform-identity of this extracellular actin should therefore be useful for further analysis of its function in serum and its potential interaction with extracellular elements such as the ECM and cell membranes in contact with serum (6,7,29). The interactions with serum proteins that bind actin, such as vitamin D-binding protein and gelsolin (30), or which interact with actin, such as plasmin (31) or angiogenin (6) may also bear investigation with these specific isoforms of actin.

PAGE identified five major polypeptides in the material eluted from the DNase-I affinity column. The p43 was identified here as actin. The other four proteins were not identified, but their sizes correspond to well-known actin-binding proteins (reviewed in (1)): vitamin D-binding protein (p58), acumentin (p65), band 4.1 (p78) and brevin or gelsolin (p93). It is known that actin is able to bind simultaneously to DNase-I and other actin-binding proteins, e.g. to vitamin D-binding protein (1). The question remains whether actin is bound to some of these proteins in the circulation or whether they form a tight complex directly on the column during affinity chromatography.

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