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Ubiquitin is a Component of Polypeptides Purified from Corpora Amylacea of Aged Human Brain

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Corpora amylacea (CA) are one of the conspicuous features of brain tissue in normal aging and neurodegenerative diseases. Quantitative protein determination of purified CA revealed a protein content of about 4% of total weight. Qualitative protein analysis revealed a broad range of polypeptides, with four being more abundant. High performance liquid chromatography (HPLC) fractionation of this protein material showed four peaks which are related to the four major polypeptides with molecular weights of 24 KD, 42 KD, 94 KD, and 133 KD. Amino acid content analysis of the 24 KD, 42 KD and 94 KD polypeptides indicated that distinct protein species are involved. N-terminal amino acid sequence analysis of the 24 KD and 42 KD polypeptides revealed in both cases homology with the N-terminal sequence of human ubiquitin.

KEY WORDS: Aging; corpora amylacea; proteins; HPLC; sequence; ubiquitin.

INTRODUCTION

Corpora amylacea (CA) are polyglucosan bodies found in neuronal processes (1) and in astrocytes (2,3), particularly near blood vessels. They are common in both aged normal brains (4-6) and brains affected with neurodegenerative diseases such as Alzheimer's disease (7-9). CA are mainly composed of glucose polymers but they also contain a small quantity of protein material, approximately 4% of the total weight of CA, (10-14). Previous studies have been mostly focused on the biochemical (10-13) and cytochemical (1-5) analysis of CA sugar components using methods which are unsuitable for qualitative analysis of their protein constituents. We have shown that the protein material of purified CA consists of several polypeptide species, with four species being more abundant (14). These polypeptides have not been identified yet.

Several accumulations of inclusions have been described in various neuropathological conditions. Because the characterization of these inclusions showed abnormal deposits of protein material (15-18), defects in protein metabolism were invoked to explain this process of deposition (19-20). Many studies have reported abnormal accumulation of ubiquitin (Ub) in inclusions such as neurofibrillary tangles in Alzheimer's disease and progressive supranuclear palsy (16,18,21-23), Lewy bodies in Parkinson's disease (16,18,21-24), Pick bodies in Pick's disease (18,21-24), and Lafora bodies in Lafora's disease (18,22,23). Ub is found associated with the protein components of these inclusions (16, 18, 22, 25, 26). Ub, a highly conserved 76 amino acid protein, is both a nuclear and a cytoplasmic protein. It is found in two forms: as a free molecule or conjugated to some other

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proteins (27-31). The apparent role of Ub is to target abnormal and short-lived normal proteins for rapid degradation through an ATP-dependent nonlysosomal proteolytic system (28-32). Ub also appears to form stable conjugates with selected normal proteins such as histones H2A and H2B in nucleus (32), lymphocyte homing receptor in plasma membrane (33,34), microtubule associated proteins and actin in the cytoskeleton (35,36), and platelet-derived growth factor receptor (37). The mode of formation of Ub containing inclusions is unknown. The identification and characterization of CA protein components would likely provide new sight into the mechanism of their formation, as well as their biological significance in normal aging and some degenerative diseases of the central nervous system. We report here the preliminary characterization of CA polypeptides following high performance liquid chromatography (HPLC) purification, and demonstrate that they contain Ub.

EXPERIMENTAL PROCEDURE

Source of Tissue. Brain tissue of elderly people (mean age 76.3 years) was removed for autopsy within 24 hrs of death and frozen at −70°C until needed. The brains were free of neurological disease.

Preparation of CA and Protein Extraction. CA were prepared according to a method described previously (14). The CA pellet was resuspended in buffer containing 0.125M Tris-HCl pH 6.8, 3% SDS, and 2% β-mercaptoethanol (βME) at room temperature for 15 minutes. The solution was then spun in a Fisher micro-centrifuge (Model 235B) at maximum speed for 15 minutes at 4°C. The supernatant was poured off in 3500 molecular weight cut-off dialysis tubing (Spectra/Por, Fisher Scientific), dialyzed four times for 30 minutes against 4L of deionized H_2O and lyophilized.

HPLC Purification of CA Polypeptides. Lyophilized CA polypeptides were solubilized in buffer containing 0.15M phosphate pH 7.0, 0.2M NaCl, 0.1% SDS, and filtered through a millex filter HV-4. Equivalents of 200 μg of protein from this solution were injected into a Waters HPLC size exclusion column 300 SW (30 cm × 75mm, protein PAK Waters) and eluted in phosphate buffer pH 7.0 at a flow rate of 0.5ml/min and a chart speed of 5mm/sec. The protein peaks were detected using a Perkin-Elmer LC95 UV/visible detector at 214nm, 2.0 AUFS. Fractions were collected from several runs, and pooled for each peak. The pooled fractions were dialyzed four times for 30 minutes against 4L of deionized H_2O and lyophilized. Samples from peak fractions were resuspended in a buffer containing 3% SDS and 2% βME and run on a 12.5% polyacrylamide gel (38) for molecular weight and purity determination. The most abundant fractions (24 KD, 42 DK, and 94 KD) were sent for amino acid content determination and sequence analysis (Protein Analysis Service, Department of Biochemistry, University of Toronto).

Amino Acid Analysis. Fractions containing polypeptides of 24 KD, 42 KD, and 94 KD were processed for amino acid analysis. One tenth of each sample was hydrolyzed in 5.7N HCl at 107°C for 22 hrs and phenol treated before being injected into a Beckman gas phase automated amino analyzer (Model 121M) fitted with a single column A/N/B.

Amino Acid Sequence Analysis. Amino-terminal sequence analyses were carried out for the 24KD and 42KD polypeptides. Nine tenths of each sample were dissolved in heptafluorobutyric acid and loaded on an Applied Biosystem gas phase sequenator (Model 470A). The collected anilothiazolone amino acids were converted to phenylthiohydantoin amino acids (PTH-amino acids). The PTH-amino acids were analyzed on an Applied Biosystem PTH-amino acid analyzer (Model 120) in line.

RESULTS

HPLC Purification of CA Polypeptides. The HPLC separation profile of polypeptides solubilized from CA showed 5 peaks (Figure 1). Peak 5 is the βME-SDS front. Fractions from peaks 1-4 were analyzed by SDS-PAGE. These fractions revealed four major polypeptide bands with molecular weights of 133 KD, 94 KD, 42 KD, and 24 KD that seem to be related to the four major bands previously reported (14). Two slight contaminant bands in the range of 50KD-60KD were also present (Figure 2). The fraction from the first peak showed a main band of 133KD, with a trace of the fraction from the second peak, the latter exhibiting a single band of 94 KD. The fraction from peak 3 showed a main polypeptide band of 42 KD and three other bands of 28 KD,

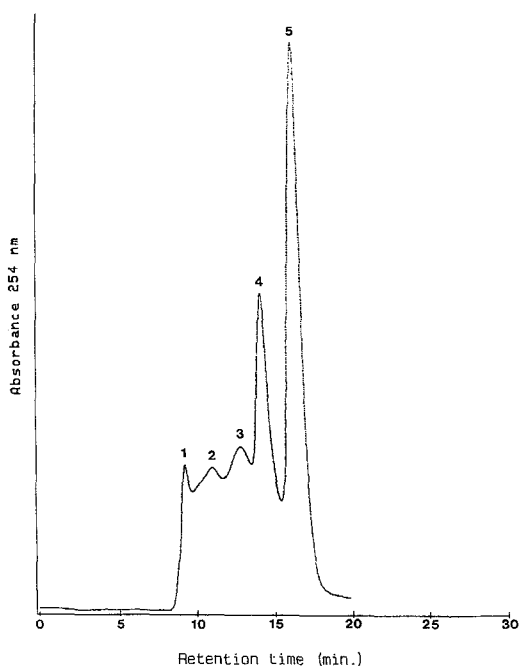

Fig. 1. HPLC profile of polypeptides solubilized from CA of aged human brain. Solubilized polypeptides in SDS-βME were chromatographed on a size exclusion column 300SW, in phosphate buffer pH 7.0. Peak 1: 133KD polypeptide, peak 2: 94KD, peak 3: 42KD, peak 4: 24KD, peak 5: SDS-βME front.

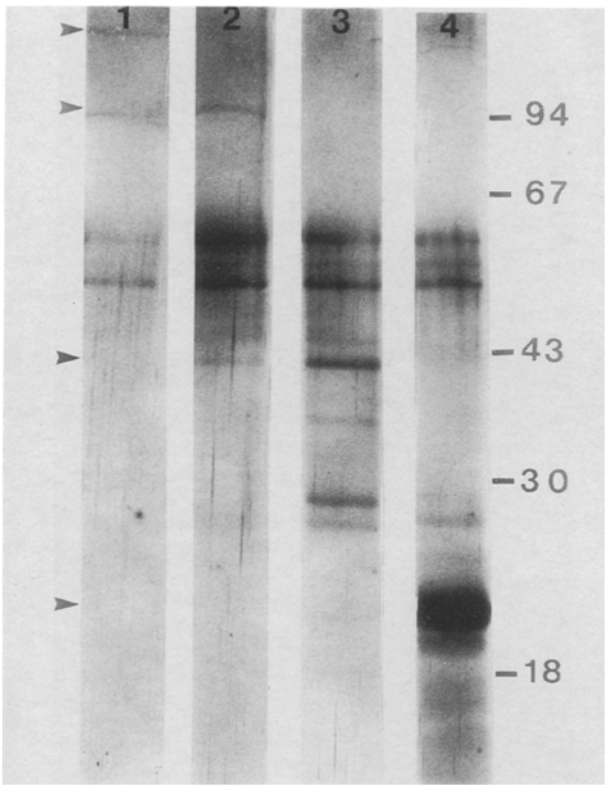

Fig. 2. SDS-PAGE of HPLC fractions of CA polypeptides. Samples from HPLC fractions were pooled and run on a 12.5% polyacrylamide gel. Lanes 1, 2, 3, 4: Samples from peaks 1, 2, 3, and 4. The arrowheads indicate the positions of the 133KD, 94KD, 42KD, and 24KD polypeptides. The numbers on the right indicate molecular weight markers.

Table I. Amino Acid Analyses of HPLC Fractions

	Molar Percent			
	Peak 4	Peak 3	Peak 2	
Amino Acid	24 KD	42 KD	94 KD	Ub
Asp	7.9	10.1	13.6	9.5
Thr	6.2	6.1	4.7	9.5
Ser	19.0	9.1	15.4	4.0
Glu	10.4	11.9	11.3	16.2
Pro	4.2	6.6	4.3	4.0
Gly	11.9	4.5	20.0	5.4
Ala	5.0	8.6	7.9	2.7
Cys	0.0	3.2	0.0	0.0
Val	3.3	4.8	3.5	5.4
Met	0.0	1.5	0.0	1.4
Ile	2.4	3.7	1.7	9.5
Leu	4.3	8.7	4.8	12.2
Tyr	3.1	3.2	1.2	1.4
Phe	3.5	4.5	2.2	2.7
His	5.4	3.2	2.1	1.4
Lys	5.5	5.4	2.9	9.5
Arg	7.0	5.2	4.2	5.4
Totals	100	100	99.8	100

29 KD, and 35 KD. The peak 4 fraction showed a single band of 24 KD.

Amino acid analsis. HPLC fractions of peaks 2, 3, and 4 were found to have different amino acid compositions (Table I). Polypeptides from fractions of peaks 2 and 4 both showed a lack of cysteine and methionine residues, suggesting that the two polypeptides may be related.

Amino acid sequence analysis. Polypeptides obtained from peak fractions 3 and 4 were submitted to amino acid sequencing and sequences were run out up to residues 30 and 17 respectively. A computer search of these sequences using the Protein Sequence Query System V4.1/4.4 revealed a homology with the NH_2-terminal of human Ub (Figure 3).

DISCUSSION

HPLC Purification of CA Polypeptide. Previous studies of CA revealed a small amount of protein (10-13). We have carried out the characterization of these protein components in order to search for proteins possibly involved in the biogenesis of CA. The HPLC purification of CA protein components showed four peaks which seem to be related to the four major polypeptides resolved on SDS-PAGE (14). Since fractions from HPLC peaks were pooled prior to SDS-PAGE analysis, the electrophoretic pattern (Figure 2) does not represent the real proportions of the 133KD, 94KD, 42KD, and 24KD polypeptides in CA. Extra bands in the SDS-PAGE profile of HPLC fraction from peak 3 (Figure 2, lane 3) may represent proteolytic by-products released upon sample handling and electrophoretic conditions and, in this respect, the 42KD polypeptide seems to be a subunit of a labile protein species.

Amino Acid Sequence Analysis. Fractions of peaks 2, 3, and 4 show a different amino acid composition, indicating that there are more than one species of protein involved. However, the absence of cysteine and methionine in fractions from peaks 2 and 4 suggests that the two fractions are related. The partial amino acid sequences to residue 17 of fractions from peaks 3 and 4 were identical and these sequences revealed homology with the NH_2-terminal of human Ub (39). Since Ub is a small protein of 8.5KD, and because of its physiological role, one could argue that the 24KD and 42KD polypeptides found in CA represent Ub conjugates. However, the apparent role of Ub in eukaryotic cells is to target abnormal proteins produced during stress for pro-

Ubiquitin* :	MET	GLN	ILE	PHE	VAL	LYS	THR	LEU	THR	GLY	10
42KD :	-	-	-	-	-	-	-	-	-	-	
24KD :	-	-	-	-	-	-	( )	-	( )	-	
:	LYS	THR	ILE	THR	LEU	GLU	VAL	GLU	PRO	SER	20
:	-	ALA	-	ALA	-	-	-	-	-	-	
:	-	( )	-	( )	-	( )	-				
:	ASP	THR	ILE	GLU	ASN	VAL	LYS	ALA	LYS	ILE	30 ... GLY 76
:	-	VAL	-	-	-	-	-	-	-	-	

Fig. 3. Amino-terminal sequences of the 42 KD and 24 KD CA polypeptides compared to that of human Ub. The homology is shown to 30 residues for the 42 KD and to 17 residues for the 24KD. Dashes indicate matching residues. Parentheses represent low yield threonine signals.
* Sequence of human Ub from Schlesinger and Goldstein (39).

teolysis (31,32). The formation of such abnormal proteins induces the synthesis of Ub (32). Therefore, the presence of Ub conjugates could be explained by the formation of altered proteins, which in turn could be attributed to either an increased frequency of unusual posttranslational modifications or a sustained physiological stress related to the aging process, if not to a combination of the two events. Ub has been found in several inclusions in neurodegenerative conditions (17-27). These findings and the results presented here suggest that stressful conditions induce similar responses in normal aging and neurodegenerative diseases. Immunochemical studies are in progress to fully characterize the protein components of CA and to identify the ub acceptor proteins found in the brain during the aging process.

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