

ACID-STABLE SERINE PROTEINASE INHIBITORS IN THE URINE OF ALZHEIMER DISEASE SUBJECTS

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SUMMARY

A comparative study of the levels of acid-stable proteinase inhibitors (kallikrein and trypsin inhibitors) in the urine of healthy and Alzheimer subjects, of both sexes, has been performed. A preliminary characterization of the purified inhibitors indicates that the urinary antitryptic activity is accounted for by the presence of the well known Urinary Trypsin Inhibitor (UTI) while an apparently new molecule appears to be responsible for the antikallikrein activity. The urinary levels of kallikrein inhibitors are very similar in healthy and sick subjects while the levels of trypsin inhibitors appear significantly increased in Alzheimer subjects of both sexes. The data presented here support the hypothesis that unpaired proteolytic processes could be involved in the pathogenesis of Alzheimer's disease and suggest that the levels of urinary acid-stable inhibitors may prove to be useful markers of the disease.

KEY WORDS Proteinase inhibitors Alzheimer disease Proteolysis

INTRODUCTION

Imbalance in the dynamic equilibrium between active proteinases and their specific inhibitors is suspected to be a pathogenic factor for many human diseases (Laskowski and Kato, 1980; Fritz and Wunderer, 1983; Travis and Salvesen, 1983; Jochum *et al.*, 1986).

A particularly significant association appears to be that between some malignancies and the expression of low molecular weight trypsin inhibitors such as the Pancreatic Secretory Trypsin Inhibitor (PSTI) (Matsuda *et al.*, 1983; Huhtala *et al.*, 1982; Ogawa *et al.*, 1987; Tomita *et al.*, 1990) and the so called Acid Stable Trypsin Inhibitor (ASTI) (Okumichi *et al.*, 1984; Onitsuka *et al.*, 1985; Sumi *et al.*, 1987; Yoshida *et al.*, 1989). The latter, a multipotent inhibitor found in several biological fluids, including urine, corresponds to the Urinary Trypsin Inhibitor (UTI) (Shulman, 1955; Gebhard and Hochstrasser, 1986) and can be obtained as a product of acid, or enzymatic, hydrolysis from the large plasma protein Inter- α -Trypsin Inhibitor (ITI) (Gebhard and Hochstrasser, 1986). Owing to its antifibrinolytic activity, UTI appears to play an important role in the metabolism of tumour cells and levels are increased in the urine of cancer patients (Chawla *et al.*, 1978; Onitsuka *et al.*, 1985).

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Recently a role has been proposed for abnormalities of proteolysis in the aetiology of Alzheimer's disease, a degenerative condition, mainly characterized by the presence of neuritic plaques in several brain regions (Abraham and Potter, 1989). It has been suggested that these proteinaceous deposits could originate from an aberration of the proteolysis in the brain (Masters *et al.*, 1985). Supportive evidence has been deduced from the finding, in the neuritic plaque, of the serine proteinase inhibitor α_1 -antichymotrypsin (Abraham *et al.*, 1988) and a 57 residue domain resembling the kallikrein inhibitor, aprotinin or "Kunitz inhibitor" (Fritz and Wunderer, 1983) within the precursor of the β -amyloid protein (Ponte *et al.*, 1988; Tanzi *et al.*, 1988; Kitaguchi *et al.*, 1988), the most abundant protein component of the plaque. Recently, it has been reported that, in proteolytic processing of the β -amyloid precursor, a proteinase very similar to cathepsin G could be involved (Razzaboni *et al.*, 1992). This enzyme which is expressed in astrocytes (Razzaboni *et al.*, 1992) in intimate contact with the neuritic plaque, could be regulated by UTI-like inhibitors (Gebhard and Hochstrasser, 1986) and kallikrein inhibitors (Razzaboni *et al.*, 1992).

We have recently found that acid treated human urine contains antiproteolytic activities against kallikrein (Eleuteri *et al.*, 1994), a serine proteinase which appears to play an important role also in cerebral function (Kizuki *et al.*, 1994). We have, therefore, undertaken a comparative study of the levels of acid-stable proteinase inhibitors (kallikrein and trypsin inhibitors) in the urine of healthy and Alzheimer subjects.

MATERIALS AND METHODS

Chemicals

Bovine trypsin, and the synthetic substrate N- α -Benzoyl-DL-Arginine-p-Nitroanilide (BzArgNan) were obtained from Sigma Chemical Co (USA). Porcine pancreas kallikrein and aprotinin were a kind gift of Bayer AG (Germany). The synthetic substrate S-2266 used for measuring the kallikrein activity was obtained from Kabi Vitrum (Sweden). Antibodies anti-human Inter- α -Trypsin Inhibitor were obtained from Dakopatts (Denmark) and utilized without further purification. Polyclonal antibodies against aprotinin, raised in rabbits, were kindly supplied by Bayer AG (Germany). Sep-Pak C₁₈ cartridges were obtained from Millipore (USA), and prepacked HiTrap™ affinity columns (1 ml volume) from Pharmacia (Sweden). All other chemicals were of analytical grade.

Urine samples

Urine samples, collected in the morning, were obtained from healthy or Alzheimer subjects of both sexes (five males and five females) of comparable age, and were treated as soon as possible after collection. Quantitation of the inhibitors was performed in triplicate on three different samples for each subject considered. Dementia was diagnosed according to the criteria of the Diagnostical Statistical Manual of mental disorders (DSM-III-Revised) (American Psychiatric Association, 1980). The subjects were classified as affected by Alzheimer's disease when the criteria reported by the NINCDS-ADRDA work group were satisfied (McKhann *et al.*, 1984).

Affinity chromatography

Porcine pancreas kallikrein, or bovine trypsin were immobilised on HiTrap™-NHS activated columns according to the suppliers' instructions.

Isolation of the inhibitors

Isolation of the acid-stable urinary inhibitors was carried out taking advantage of a procedure which allows the quantitative recovery of small amounts of active inhibitors in biological fluids essentially based on affinity- and reversed phase-HPLC chromatography as major purification tools (Fioretti *et al.*, 1993) (see Scheme 1).

HPLC

The HPLC analyses were performed with a System Gold apparatus (Beckman, USA) using a reversed phase (RP) column Supelcosil LC 18-DB, equipped with a guard column, both obtained from Supelco (USA). The column was equilibrated in 0.1% trifluoroacetic acid (TFA) in water (solvent A) and eluted with a gradient obtained by mixing solvent A with a solution of 4:1 (v/v) acetonitrile-2-propanol in 0.1% TFA (solvent B) at a flow-rate of 1 ml/min. The inhibitor samples were prepared by dissolution with solvent A just before use. Absorbance of the eluted fractions was recorded at 254 nm. Chromatographic data were analyzed with the Gold software.

Concentration of the inhibitors

The concentration of the urinary inhibitors was determined by titration with an aprotinin standardized trypsin solution, as previously reported (Fioretti *et al.*, 1993).

Antiproteolytic activity

The antiproteolytic activity of the urinary inhibitors, in the HPLC eluates, was determined by measuring their antitryptic or/and antikallikrein activity as previously reported (Fioretti *et al.*, 1993). The presence of organic solvents, which affects the proteolytic activity, was taken into account adding, in the reference test, an eluate volume from a run performed without the inhibitor.

Immunochemical characterization of the inhibitors

Cross-reactivity between polyclonal antibodies raised against bovine aprotinin, or human ITI, and the urinary inhibitors was assayed as previously reported (Fioretti *et al.*, 1984; Fioretti *et al.*, 1985) measuring the ability of the antibodies to remove the antitryptic activity of the inhibitors.

RESULTS

The experimental procedure utilized for the isolation of the acid-stable urinary proteinase inhibitors is summarized in Scheme 1. Usually, 200–250 ml of freshly collected urine was acidified by slow addition, under stirring, of a concentrated solution (50%) of trichloroacetic acid to a final TCA concentration of 2.5%. The supernatant obtained after centrifugation, was brought to pH 8.6 with concentrated NaOH solution and applied to a 1 ml prepacked HiTrap™-kallikrein column, for isolation of the kallikrein inhibitors, or, alternatively, to a 1 ml HiTrap™-trypsin column for the isolation of the trypsin inhibitors. Both columns were washed with the equilibrium buffer (200 mM Tris-HCl pH 8.6 containing 20 mM CaCl₂) and eluted with a solution of HCl at pH 2.0. In both cases, the fractions showing antiproteolytic activity (measured as antitryptic activity) were pooled, neutralized and loaded on a Sep-Pak C₁₈ cartridge. The cartridge was eluted with a mixture obtained by mixing 60% of Solvent A and 40% of Solvent B

Scheme 1. Procedure adopted for the isolation of the urinary acid-stable proteinase inhibitors.

Freshly collected urine	
Trichloroacetic acid precipitation	
Kallikrein affinity chromatography	Trypsin affinity chromatography
bound material	bound material
RP-Chromatography (Sep-Pak C ₁₈ + C ₁₈ HPLC column)	RP-Chromatography (Sep-Pak C ₁₈ + C ₁₈ HPLC column)
Kallikrein inhibitors	Trypsin inhibitors

(see Materials and Methods). This fraction (about 3.0 ml) was utilized for the quantitative determination of the inhibitors in the urine samples (see Table 1). The determination was carried out using a titration with an aprotinin-standardized trypsin solution (see Material and Methods) taking advantage of the high affinity ($K_d < 10^{-11}M$) shown by both kinds of urinary inhibitors for the enzyme (see also below). Further information on the nature of the inhibitors recovered from the affinity columns was obtained through an HPLC-reversed phase (RP) analysis and a preliminary molecular characterisation. The fraction obtained from the Sep-Pak C₁₈ cartridge was concentrated to dryness, dissolved in 50 μ l of Solvent A and then loaded on a RP-C₁₈ column, equilibrated and eluted as reported in the Material and Methods section. Figure 1 reports the elution profile and the antiproteolytic activity, determined against kallikrein and trypsin, of the fractions eluted from the C₁₈ column when the material recovered from the kallikrein affinity column was chromatographed. In this case, the fractions showing antikallikrein activity (hereafter termed "kallikrein inhibitors") are eluted from the column at a concentration ranging between 30% and 40% of Solvent B, and both the elution profile and the antikallikrein activity appear very similar when the urine of Alzheimer (Figure 1B) or control subjects (Figure 1A) was analyzed. The kallikrein inhibitors also show antitryptic activity and their antiproteolytic activity is unaffected either by the presence of polyclonal antibodies raised against the bovine aprotinin or by anti-human ITI antibodies which are immunologically identical to anti-UTI antibodies (Gebhard and Hochstrasser, 1986) (see above).

These results prompted further investigation of the nature of urinary kallikrein inhibitors. The fraction recovered from the C₁₈ column was subjected either to SDS-polyacrylamide gel electrophoresis in non reducing conditions (Laemmli, 1970) or to gel permeation chromatography on a Superdex 75 HR 10/30 column (Pharmacia, Sweden).

Table 1. Urine content of acid-stable, low molecular weight, proteinase inhibitors in Alzheimer and healthy subjects.

	Alzheimer		Control	
	Male	Female	Male	Female
Age (years)	80–90	80	80–90	80
Kallikrein inhibitors ($\mu\text{g}/\mu\text{l}$)	10 \pm 1	8 \pm 1	9 \pm 1	10 \pm 1
Trypsin inhibitors ($\mu\text{g}/\mu\text{l}$)	480 \pm 5	230 \pm 5	143 \pm 4	130 \pm 5

Inhibitor content was determined on the basis of antitryptic residual activity using an aprotinin standardized trypsin solution. Determinations were performed in triplicate on the fractions recovered from the Sep-Pak C₁₈ cartridge (see Materials and Methods).

Both analyses showed that this fraction contained several proteins and that the antikallikrein activity was associated to a protein showing an apparent molecular weight of about 24 KDa. The complete characterization of this inhibitor is in the progress.

When the material recovered from the immobilized trypsin column was chromatographed on the RP-C₁₈ HPLC column under the conditions reported, the antiproteolytic activity was found in fractions eluted at a concentration ranging between 30% and 80% of Solvent B (Figure 2). As in the previous case, these fractions were assayed for anti-kallikrein activity and for their cross reactivity towards anti-aptopinin and anti-ITI antibodies. The results obtained clearly indicate that, in this case, two kinds of trypsin inhibitors are present in the C₁₈ elute: i) a first one eluted between 30% and 40% of solvent B, accounting for the presence of the kallikrein inhibitors (see above) which shows also antitryptic activity and a second one (hereafter termed the “trypsin inhibitors”) eluted between 40% and 80% of solvent B, which is inactive against kallikrein and recognizes anti-human-ITI antibodies (Figure 3). Also in this case, both the elution profile and the antiproteolytic activity of the fractions eluted when urine of Alzheimer (Figure 2B) or control subjects (Figure 2A) was subjected to HPLC analysis, appear very similar. The urinary antitryptic activity is associated, as expected, with multiple protein peaks showing an apparent molecular weight of 30KDa, 24KDa and 18KDa respectively, most likely arising from a proteolytic fragmentation of the native Inter- α -Trypsin Inhibitor. As can be seen in Table 1, where the results obtained from this study are summarized, striking differences in the urinary levels of the kallikrein and trypsin inhibitors were observed. The urine content of kallikrein inhibitors (about 10 $\mu\text{g}/\text{l}$) was very similar for healthy and Alzheimer subjects of both sexes, while in ill subjects a marked increase in the levels of the trypsin inhibitors was observed. As can be seen in Table 1 a mean content of 135 μg of trypsin inhibitors per litre of urine was found in control subjects (150 $\mu\text{g}/\text{litre}$ for males and 120 $\mu\text{g}/\text{litre}$ for females). This value is elevated 2–3 fold in the urine of Alzheimer patients

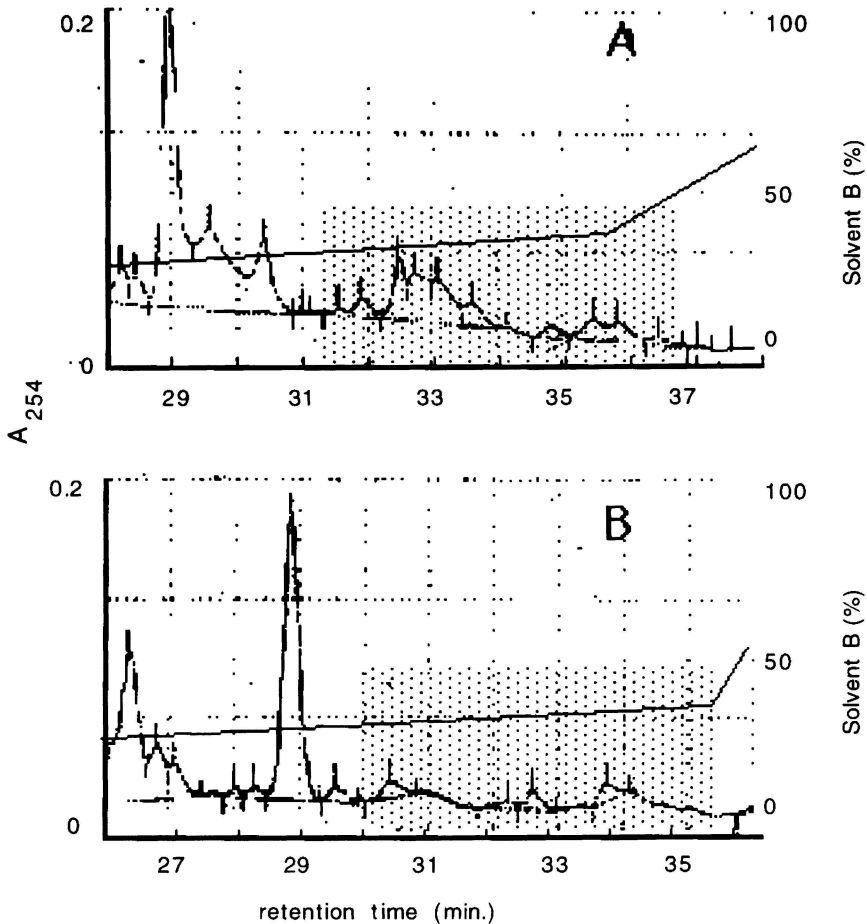


Figure 1. HPLC elution profile and antiproteolytic activity of the urinary kallikrein inhibitors isolated from the urine of Alzheimer (B) or control subjects (A). Column: Supelcosil LC₁₈-DB. Flow-rate: 1.0 ml/min. Detection wavelength: 254 nm. The shaded area accounts for the fractions showing antiproteolytic activity determined against kallikrein and trypsin. For other details see Materials and Methods.

DISCUSSION

A correct balance between proteolytic and antiproteolytic activities appears important for many physiological processes, those occurring at brain level included. Proteinases and proteinase inhibitors have, in fact, been involved in several aspects of neurogenesis and in glial function (Pittman, 1984; Krystosek and Seeds, 1984; Pittman, 1985; Pittman, 1987). A more direct involvement has recently been reported for the serine proteinases kallikrein (Kizuki *et al.*, 1994) and cathepsin G (Razzaboni *et al.*, 1992). Immunoreactivity for glandular kallikrein was found in both the cerebral cortex and the brain stem of rats and appeared to be concentrated in the neuronal cell bodies, indicating, for this protease, an involvement in neuron function (Kizuki *et al.*, 1994). In addition, a marked decrease in kallikrein activity has been observed in the brain of Alzheimer subjects

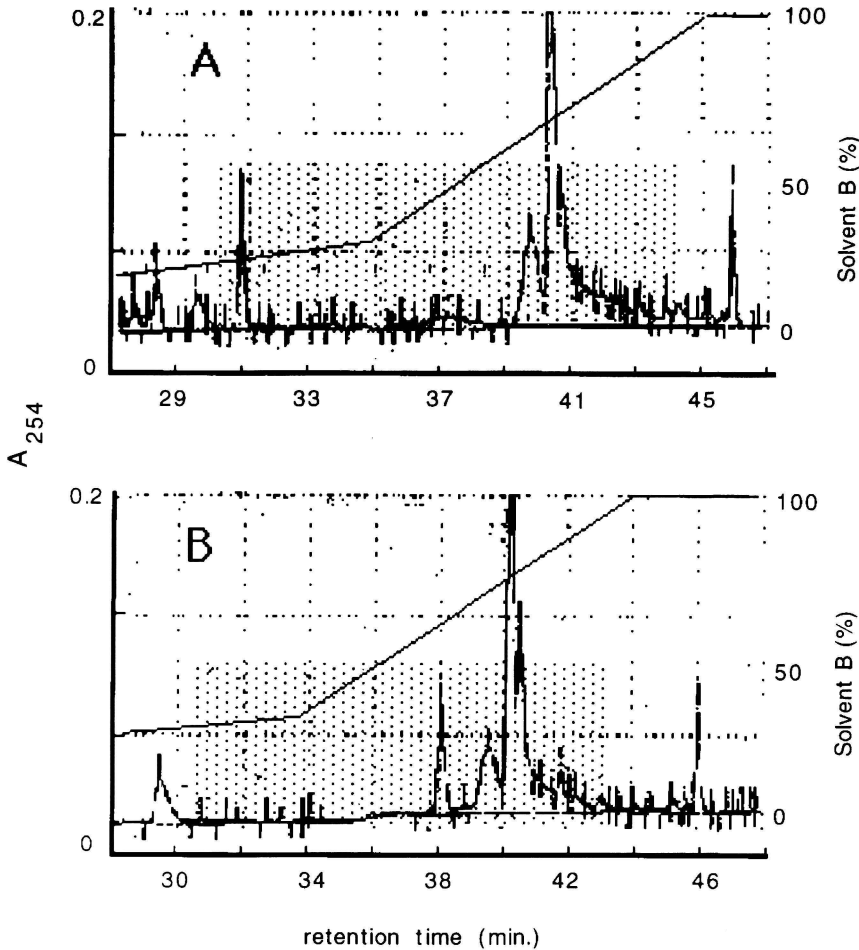


Figure 2. HPLC elution profile and antiproteolytic activity of the urinary trypsin inhibitors isolated from the urine of Alzheimer (B) or control subjects (A). Column: Supelcosil LC₁₈-DB. Flow-rate: 1.0 ml/min. Detection wavelength: 254 nm. The shaded area accounts for the fractions showing antitryptic activity. For other details see Materials and Methods.

(Aoyagy *et al.*, 1990) as well as in old senescence-accelerated mice (Kizuki *et al.*, 1994). A cathepsin G-like activity has been partially purified from the normal human and monkey brain and from the brain of Alzheimer subjects (Razzaboni *et al.*, 1992); this proteolytic activity appears to be sensitive to inhibition by the kallikrein inhibitor (aprotinin) and is localized in astrocytes, glial cells surrounding the neuritic plaque (Razzaboni *et al.*, 1992). Owing to this localization it has been hypothesized that this proteinase could be involved in processing the β -amyloid precursor and hence could influence formation and deposition of the β -amyloid. We now show that, in human urine, acid-stable proteinase inhibitors showing antikallikrein or antitrypsin activity are present and that their levels, at least for the trypsin inhibitors, are increased in subjects with Alzheimer's disease. Both kallikrein- and trypsin inhibitors show a broad specificity towards proteolytic enzymes and are active against proteinases apparently involved in

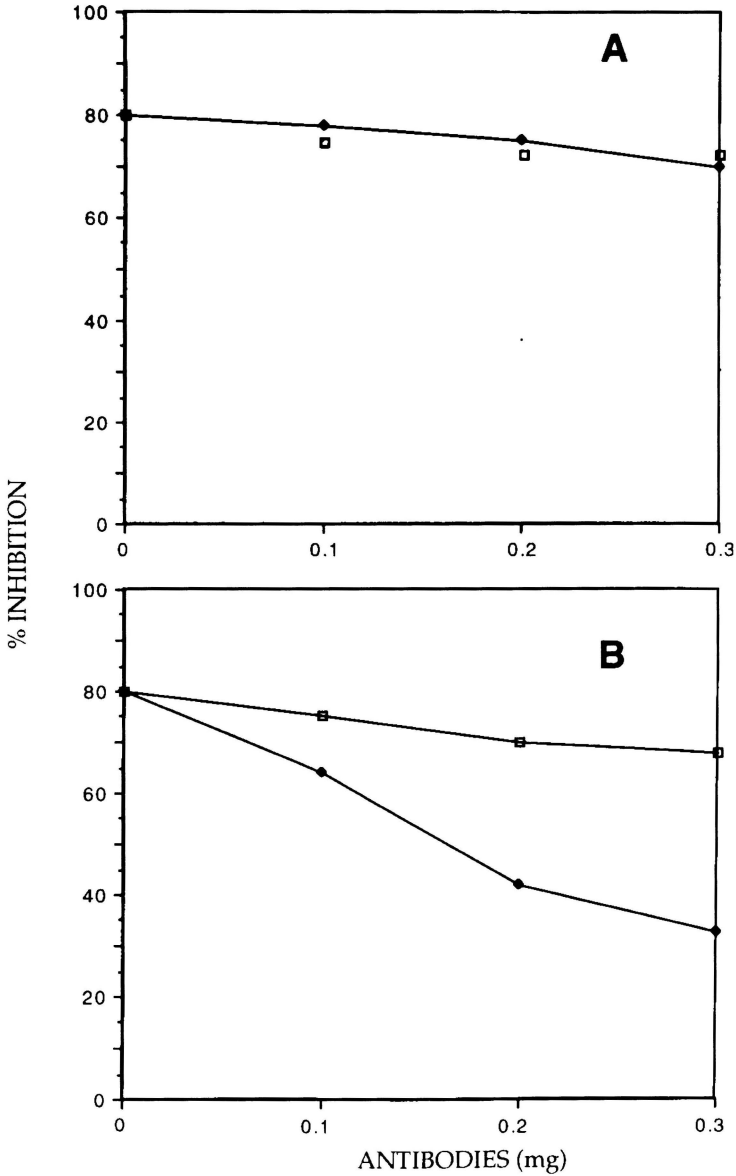


Figure 3. Effect of increasing quantities of anti-aprotinin antibodies (□) and anti-Inter- α -Trypsin Inhibitor antibodies (◆) on the antiproteolytic activity of the urinary trypsin inhibitors eluted from the RP-C₁₈ column at a concentration of solvent B ranging between 30% and 40% (A) and between 40% and 80% (B). The effect was followed measuring the ability of the antibodies to remove the antitryptic activity of the inhibitors as previously reported (Fioretti *et al.*, 1984; 1985).

the function of nervous system cells such as kallikrein and cathepsin-G (the UTI-like inhibitors). In addition, the urinary proteinase inhibitors are characterized by a relatively low molecular weight and appear potentially able to cross the blood-brain barrier. UTI-like inhibitors, deriving from proteolytic processing of the plasma protein Inter- α -

Trypsin Inhibitor, are circulating molecules found in many biological fluids. In conclusion, the finding of increased levels of UTI-like inhibitors in urine of Alzheimer's disease patients seems to support the hypothesis that aberrant proteolytic processes could be involved in the pathogenesis of this neurological disorder. Nevertheless the results presented here are not sufficient for assigning a specific role to acid-stable proteinase inhibitors in degenerative phenomena of the nervous system, but can be seen as a starting point for further studies. In particular, it could be important to examine a larger sample of Alzheimer subjects and to perform a similar analysis on biological fluids (such as the cerebral spinal fluid) in a closer contact with the brain tissue.

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