A new simple enzyme assay for pre- and postnatal diagnosis of infantile neuronal ceroid lipofuscinosis (INCL) and its variants

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Short reports

A new simple enzyme assay for pre- and postnatal diagnosis of infantile neuronal ceroid lipofuscinosis (INCL) and its variants

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Abstract

Palmitoyl-protein thioesterase (PPT) deficiency was recently shown to be the primary defect in infantile neuronal ceroid lipofuscinosis (INCL). The available enzyme assay is complicated and impractical for diagnostic use and is, in practice, unavailable. We have developed a new fluorimetric assay for PPT based on the sensitive fluorochrome 4-methylumbelliferone. This PPT assay is simple, sensitive, and robust and will facilitate the definition of the full clinical spectrum associated with a deficiency of PPT. PPT activity was readily detectable in fibroblasts, leucocytes, lymphoblasts, amniotic fluid cells, and chorionic villi, but was profoundly deficient in these tissues from INCL patients. Similarly, a deficiency of PPT was shown in patients with the variant juvenile NCL with GROD. These results show that rapid pre- and postnatal diagnosis can be performed with this new enzyme assay for PPT.

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Keywords: infantile neuronal ceroid lipofuscinosis; CLN1; palmitoyl-protein thioesterase; enzyme analysis

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The neuronal ceroid lipofuscinoses (NCL) are a group of inherited, progressive encephalopathies characterised by lipofuscin-like inclusions in various tissues. At least four distinct clinical forms have been described. The infantile form of neuronal ceroid lipofuscinosis (INCL, Santavuori-Haltia disease, MIM 256730) is an early onset, neurodegenerative disease characterised by psychomotor regression, seizures, and progressive macular degeneration leading to blindness after the first year. Severe brain atrophy is observed by neuroimaging. The inheritance of INCL is autosomal recessive and the classical form is frequent in Finland (1 in 13 000 births) and rare in the rest of the world. Electron microscopical investigations of skin or rectal biopsies show the characteristic granular osmiophilic deposits (GROD).

Until recently, the confirmation and classification of the subtypes was based entirely on the appearance of the pathological inclusion bodies, being GROD, fingerprint-, or curvilinear structures.[1] During the last few years rapid advances have been made in discovering the primary defects in various forms of NCL. In 1995 the gene involved in juvenile NCL (CLN3) was identified by the Batten disease consortium.[2] The corresponding protein is an integral lysosomal membrane protein,[3] but its biological role is still unknown. In 1997, Sleat *et al*[4] showed that patients with the classical form of late infantile NCL had mutations in a gene (CLN2) which had homology to bacterial pepstatin insensitive carboxypeptidases. A corresponding carboxypeptidase was found to be deficient in brain extracts from late infantile NCL patients.

In 1995, Vesa *et al*[5] reported a deficiency of palmitoyl-protein thioesterase (PPT) in patients with the infantile form of NCL and found mutations in the corresponding CLN1 gene. PPT is a typical soluble lysosomal hydrolase which is routed to lysosomes through the mannose-6-phosphate signal.[6] This classifies INCL as a genuine lysosomal storage disorder and has ended the debate about whether infantile NCL belongs to the group of lysosomal storage disorders.

The published enzyme assay for PPT[7] uses a [^{3}H]-S-palmitoyl-H-Ras protein as substrate. The substrate has been made in a baculovirus expression system labelled in situ with [^{3}H]-palmitate followed by elaborate purification. PPT hydrolyses the thioester linkage between the palmitoyl group and the sulphur atom of particular cysteine residues from the H-Ras protein. Unfortunately, this sophisticated assay is not practical for the diagnostic enzyme laboratory. Recently, a modification of this assay using a [^{3}H]-S-palmitoyl-peptide has been published.[8] We present here a novel and simple assay for PPT based on the widely used fluorochrome 4-methylumbelliferone, show its application in rapid postnatal enzyme analysis, and report the first, retrospective, prenatal enzyme analyses of cases affected with INCL.

Patients and methods

Fibroblasts and leucocytes were obtained from patients with the typical clinical signs and

symptoms of INCL. The diagnosis was confirmed by the finding of GROD in appropriate tissues.

The synthetic substrate for palmitoyl-protein thioesterase (PPT) was prepared by linking 4-methylumbelliferyl-6-thio-β-D-galactopyranoside (MU-6-thio-β-galactoside) to palmitate through a thioester bond (MU-6S-Palm-βGal; available from Moscerdam Substrates, for inquiries O P van Diggelen). Total leucocytes were isolated from heparinised blood as described previously[9] and frozen until use. Skin fibroblasts were cultured according to routine procedures in Ham's F10 medium supplemented with 10% fetal bovine serum and antibiotics. The cells were harvested with trypsin seven days after the last subculture and stored at −70°C until use. Lymphoblasts were prepared by transforming peripheral blood lymphocytes with Epstein Barr virus, according to standard procedures. Homogenates were prepared by sonication of cell material in water. For the standard one step PPT assay, reaction mixtures consisted of 10 µl homogenate (2 µg protein for fibroblasts, lymphoblasts, and amniocytes; 5 µg for chorionic villi and leucocytes) and 20 µl substrate solution containing 0.64 mmol/l MU-6S-Palm-βGal, 15 mmol/l dithiothreitol (DTT), 0.375 % (w/v) Triton X-100, and 0.1 U β-galactosidase from *Aspergillus oryzae* (Sigma) in McIlvain's phosphate/citrate buffer, pH 4.0. The reaction mixtures were incubated for 17 hours at 37°C. The exogenous β-galactosidase from *Aspergillus* was necessary since the endogenous β-galactosidase activity was insufficient to convert the reaction intermediate MU-6-thio-β-galactoside quantitatively. PPT activities were similar in acetate buffer and phosphate/citrate buffer. Mg^{2+} and EDTA (7 mmol/l) had no effect on the PPT activity.

In the two step assay, the first incubation was identical to the standard assay, except that *Aspergillus* β-galactosidase was not added. After the first incubation of 17 hours at 37°C, the samples were boiled for two minutes to stop the PPT reaction. After cooling, 0.1 U of *Aspergillus* β-galactosidase was added and the second step was incubated for six hours at 37°C to hydrolyse the reaction intermediate MU-6-thio-β-galactoside. In the experiment to determine the optimum pH, buffer was added to adjust the pH to 4.5. Finally, all enzymatic reactions were terminated by the addition of 200 µl of 0.5 mol/l $Na_2CO_3/NaHCO_3$, pH 10.7 and the fluorescence of 4-methylumbelliferone (MU) was measured in a FluoroCount (Packard) fluorimeter. Protein was determined as described previously.[9]

Results

The potential of 4-methylumbelliferyl-6-thiopalmitoyl-β-D-galactopyranoside (MU-6S-Palm-βGal) as an artificial substrate for the palmitoyl-protein thioesterase (PPT) was investigated. The liberation of MU from MU-6S-Palm-βGal cannot be accomplished by the sole action of PPT, since this enzyme only hydrolyses the palmitoyl thioester linkage, yielding the non-fluorescent reaction intermediate MU-6-thio-β-galactoside. The endogenous β-galactosidase, present in cell homogenates, was not able to hydrolyse the reaction intermediate quantitatively, but exogenous β-galactosidase from *Aspergillus* (0.1 U) could hydrolyse MU-6-thio-β-galactoside completely within six hours, albeit at a low rate (data not shown). The release of MU required the presence of a reducing agent (DTT) to prevent formation of disulphides between MU-6-thio-β-galactosides. Using the two step assay (see Patients and methods section), the optimum pH of PPT for leucocytes and fibroblasts was shown to be 4 (fig 1A). Activities at pH >6 could not be determined owing to spontaneous hydrolysis of the palmitoyl thioester bond of MU-6S-Palm-β-Gal. The pH optima for amniocytes and chorionic villi were similar (not illustrated). In fibroblasts and leucocytes from INCL patients, the thioesterase activity at pH 3.5 was almost absent but gradually increased at higher pH values, reaching control levels at pH 6. This indicated that thioesterases other than PPT can also hydrolyse the substrate at higher pHs. All further experiments were done at pH 4. The PPT assay could also be carried out in one step by adding exogenous β-galactosidase together with the substrate. In fig 1B, PPT activities in fibroblasts and leucocytes, measured in one and two steps, are compared. In the two step assay, different incubation times for the first (PPT) step were followed by an incubation period of six hours with exogenous β-galactosidase. In the one step assay, activities are underestimated if incuba-

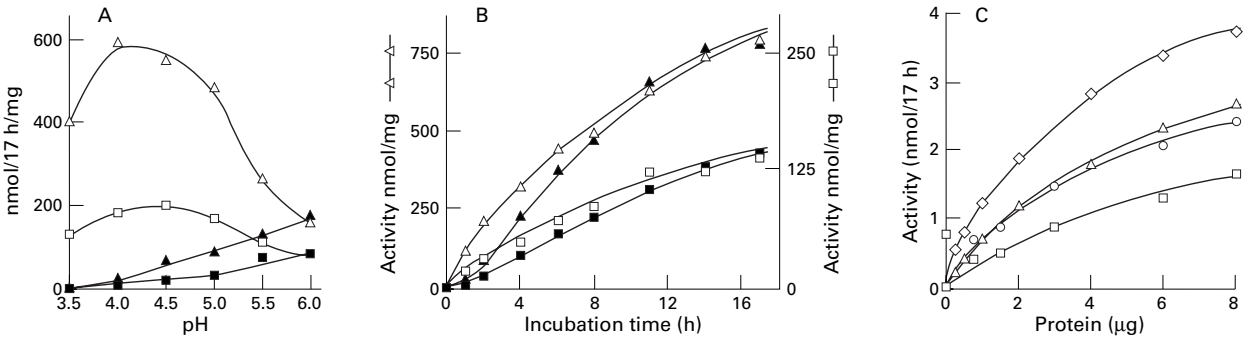

Figure 1 (A) pH dependence of palmitoyl-protein thioesterase activity. Fibroblasts from a control (open triangles) and an INCL patient (filled triangles); leucocytes from a control (open squares) and an INCL patient (filled squares). (B) Time course of palmitoyl-protein thioesterase activity in the one step assay (closed symbols) and two step assay (open symbols). Control fibroblasts (triangles) and control leucocytes (squares). (C) Protein dependence of palmitoyl-protein thioesterase activity. Control fibroblasts (triangles), control leucocytes (squares), control amniocytes (diamonds), control chorionic villi (circles). Reaction conditions as described in the Patients and methods section, except for the parameter which is varied.

Table 1 PPT deficiency in infantile and variant juvenile ceroid lipofuscinosis

	Palmitoyl-protein thioesterase activity (nmol/17h/mg)				
	Fibroblasts	*Leucocytes*	*Lymphoblasts*	*Amniocytes*	*Chorionic villi*
INCL Finnish	7–21 (n=5)	2; 10 (n=2)			
INCL non-Finnish	5–22 (n=12)	4–13 (n=4)	6 (n=1)	20 (n=1)	18 (n=1)
vJNCL+GROD		7; 9 (n=2)			
INCL heterozygotes	399–435; 640 (n=5)	99–128 (n=5)			
I cell disease	45 (n=1)				
Controls	380–850 (n=32)	90–280 (n=48)	260–450 (n=5)	410–910 (n=12)	125–520 (n=15)

tion times are less than four hours owing to the slow hydrolysis of the reaction intermediate MU-6-thio-β-galactoside by β-galactosidase. However, after an overnight incubation, the one step assay approached the "true" PPT activity as measured in the two step assay. Therefore, a one step assay with a 17 hour incubation was used in the experiments described below. Amniocytes and chorionic villi gave similar results (not shown). The PPT activity increased in a non-linear manner with the amount of protein up to 8 μg (fig 1C). As a compromise between linearity and sensitivity of the assay, we chose the fixed protein amounts as mentioned in the Patients and methods section. The apparent Km was estimated to be 0.2 mmol/l for fibroblasts and leucocytes (data not shown).

Under standard conditions PPT activity was readily detectable in control leucocytes, lymphoblasts, fibroblasts, amniocytes, and chorionic villi (table 1), whereas the activity was reduced in leucocytes and fibroblasts from obligate heterozygotes for INCL. PPT activity was profoundly deficient in fibroblasts, total leucocytes, and lymphoblasts from patients with INCL (table 1). PPT activity was also deficient in leucocytes from the variant juvenile NCL with GROD. In fibroblasts from patients with I cell disease, a multiple lysosomal enzyme deficiency, PPT was considerably reduced.

Retrospectively, a clear deficiency of PPT was shown in amniotic fluid cells (amniocytes) and chorionic villi from fetuses affected with INCL (table 1). This shows the feasibility of prenatal enzyme analysis for INCL.

Discussion

We have synthesised an artificial substrate for palmitoyl-protein thioesterase with a thioester bond between palmitate and 6-thiogalactose linked to the fluorochrome 4-methylumbelliferone (MU-6S-Palm-βGal). The specificity of this substrate was shown by the deficiency of PPT in various cell types from patients with the infantile form of neuronal ceroid lipofuscinosis and its variants. To our knowledge, thioesters between fatty acids and thio-sugars do not exist in nature and our results show that PPT does not discriminate between the compounds to which the fatty acid thioester is linked. This is consistent with the observation that PPT also hydrolyses palmitoyl-CoA.[7] Thus, PPT shares a property typical of most lysosomal enzymes, viz that it is the terminal group of the linkage hydrolysed by the enzyme that determines the specificity of the enzyme. This property has been exploited for diagnostic purposes and nearly all the lysosomal storage disorders are now diagnosed by enzyme assays using artificial substrates based on the sensitive fluorochrome 4-methylumbelliferone.

Since the primary defect in infantile NCL was reported,[5] it has become clear that classification based on age of onset and electron microscopical histopathology is no longer completely reliable and that a heterogeneous clinical phenotype of a deficiency of PPT is emerging. Das *et al*[10] showed that only half of the patients with PPT deficiency in the USA and Canada have the severe phenotype characteristic of the Finnish INCL. The availability of a simple enzyme assay will greatly facilitate the definition of the full clinical spectrum of a deficiency of PPT and it is an intriguing thought that late onset patients with PPT deficiency, with or without GROD, might be discovered by enzyme analysis. The correlation between the presence of GROD and a deficiency of PPT is good but not absolute. A patient with GROD in ganglion cells, but homozygous for a mutation in the gene for classical juvenile NCL (CLN3) has been reported.[11]

In the Finnish population, screening for just a single mutation has been an efficient means of confirming the diagnosis in 95% of the INCL patients,[5] albeit that the sensitivity is not 100%. In non-Finnish patients with a PPT deficiency, a large number of different mutations has been found,[10] indicating that screening for known mutations is not efficient in identifying the NCL subtype. In contrast, the present assay of functional PPT screens for all known and unknown mutations leading to a PPT deficiency. Therefore, enzyme analysis should always precede mutation analysis in the process of establishing the diagnosis in new patients. In fact, enzymatic confirmation of PPT deficiency in Dutch patients suspected of having INCL has initiated a screening programme for mutations in the CLN1 gene in The Netherlands.

Our fluorogenic PPT assay is much more convenient than the assays using purified [^{3}H]-palmitoylated-H-Ras protein[7] or a [^{3}H]-palmitoylated peptide.[8] We have shown that the present PPT assay is a straightforward way to confirm the diagnosis of patients suspected of having INCL. We have also shown that it is possible to see a deficiency of PPT in fetal samples.

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