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Tripeptidyl peptidase I: a minireview

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Many proteases are now regarded as marker enzymes for different malignant, neurodegenerative, immunological and other diseases. Amongst them, a considerable interest attracts the lysosomal serine-type protease tripeptidyl peptidase I (TPP I). Genetically determined TPP I deficiency is known to cause the late infantile neuronal ceroid lipofuscinosis. Aberrant expression of the enzyme is reported for a number of diseases and it is proposed as a specific marker for breast carcinoma. The purpose of the present review is to summarize the existing data about TPP I, its diagnostic and prognostic value for different diseases as well as the most recent methods for the *in situ* visualization of the enzyme activity.

Key words: tripeptidyl peptidase I, genetic diseases, enzyme markers, enzyme histochemistry

Nomenclature and substrate specificity

Tripeptidyl peptidase I (TPP I; EC 3.4.14.9) is a serine-type lysosomal protease with an unusual catalytic triad of serine, aspartic acid, glutamic acid, allowing the enzyme to be active at pH < 7 [34]. The enzyme has two types of proteolytic activity: 1) weak endopeptidase activity of pH optimum 3.0, which is probably connected with the autoactivation process [8] and 2). strong exo-peptidase activity at pH 4.5 [30]. As an exopeptidase TPP I cleaves tripeptides from oligo- and polypeptides. It has been shown that the hog enzyme is highly specific towards peptides, possessing Gly-Pro-Met amino acid sequence at their N-terminal [19], the rat enzyme can cleave off Gly-Pro-Met and Ala-Ala-Phe tripeptides [31], whereas the sequence Ala-Ala-Phe is the most favourable for the human TPP I [21]. Recent comprehensive studies on the enzyme specificity [30] have proved that the human TPP I acts preferably on Ala-Ala-Phe triplets, but also show that it is able to cleave off tripeptides, possessing the atypical amino acid norleucine at P1 position, such as Arg-Nle-Nle. Natural substrates of TPP I are not clearly established, but it has been reported that the enzyme participates in the hydrolysis of collagen [21] and peptide hormones like glucagons, angiotensins II and III [31], substance P [12] and neuromedine B [6]. Being a serine protease, TPP I is easily inhibited

by 3,4-dichloroisocoumarin and diisopropylfluorophosphate [16]. The activity of TPP I is suppressed efficiently and specifically by the tripeptide analogue of the substrate Ala-Ala-Phe-chloromethylketone [21, 31].

Synthesis and processing

The gene, encoding TPP I in humans has been carted at 11p15 [25]. It encodes a sequence of 563 amino acids, including a 19 amino acid signal sequence and 176 amino acids removed during the maturation process. The mature enzyme molecule contains 368 amino acid moieties [17]. The purified enzyme from human osteoclastoma [21], rat spleen and kidney [6, 31], bovine brain [12] and human brain [18] represents a molecule of 46-48 kDa. However, the non-denaturating PAGE and gel-filtration of the rat TPP I showed a molecular weight of 280-290 kDa, which presumes that at least the rat enzyme consists of 6 identical sub-units [6]. The enzyme is synthesized in the rough endoplasmic reticulum as a pre-proenzyme of molecular weight 68 kDa. The signal polypeptide is removed simultaneously with the translocation to cis-Golgi and the glycosylation at asparagine moieties. During its consecutive transfer to cis-Golgi, trans-Golgi and lysosomes the proenzyme becomes a subject of different modifications, including glycosylation and transformation of carbohydrate chains. The final maturation of TPP I takes place in the lysosomes and is connected with a proteolytic cleavage by unknown serine protease, sensitive to 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBFS) [7]. In vitro studies of Golabek et al. [8] however show, that under pathological conditions TPP I is able of auto-activation of intramolecular mechanism.

Genetically determined TPP I deficiency

It has been shown that the gene, encoding TPP I coincides with CLN2, mutations of which are known to cause the late-infantile form of neuronal ceroid lipofuscinosis (LINCL) [24, 25]. Fifty-two mutations of different nature (deletions, insertions, etc.) and 22 polymorphysms are now known to cause LINCL [20]. LINCL is one of the most common neurodegenerative disorders in Europe. For example, in UK six cases are diagnosed per year, but many cases are described also in Canada, USA, Russia and China. It is characterized by seizures, myoclonal jerks and developmental regression in the early stage, followed by a visual failure and an early death at puberty. A small number of mutations are associated with a later disease onset and slower symptom progression. Sleat et al. [26] have described several atypical phenotypes of LINCL, represented by a delayed onset at around eight years of age and death in the fourth or fifth decades. Morphologically, LINCL is characterized by fluorescent curvilinear profiles of lypopigments that aggregate in membrane-bound lysosomal residual bodies, called curvilinear bodies. However, in the cases of mutations in CLN2, causing later onset of LINCL, curvilinear bodies may not be pure and contain other types of deposits [33]. Also, other types of neuronal ceroid lipofuscinosis (NCL), caused by mutations in other genes (CLN 3, 5, etc.) may have the same curvilinear profiles. A considerable part of the auto-fluorescent material, which deposits in the lysosomes during all the forms of NCL, including LINCL has been identified as the sub-unit c of mitochondrial ATP synthase [22]. Recent experiments of Tlan et al. [30] on the TPP I substrate specificity however, have shown that the sub-unit c of mitochondrial ATP synthase is a poor substrate of TPP I. So, the accumulation of this protein in the lysosomes may be considered as a secondary effect of the genetically determined TPP I deficiency and can not serve as a tool for

the diagnosis of LINCL. The LINCL diagnosis now relies on the enzyme analysis for TPP I activity and a subsequent confirmation by CLN2 mutation analysis [20]. Different enzyme assays have been proposed for the diagnosis of CLN2 deficiency:

1) A diagnostic assay, based on the ability of specimens from healthy individuals but not patients with LINCL to degrade hemoglobin into trichloroacetic acid-soluble products in the presence of aspartyl and cisteinyl proteases inhibitors [28].

2) A specialized assay that entails monitoring cleavage of a chromophore-labeled peptide after high-performance liquid chromatography (HPLC) [11].

3) TPP I assay using the substrate Ala-Ala-Phe-4-methyl coumarylamide, applied to cultured skin fibroblasts with the TPP I activity of LINCL fibroblasts being around 5 % of healthy controls [1, 32].

Modern enzyme analyses are based on the substrate Ala-Ala-Phe-4-methylcoumarylamide [29], which can be used for biochemical assays of TPP I activity in tissue homogenates and cell suspensions. No cytochemical substrate has been proposed thus far.

It has been shown that TPP I can be secreted from cells, over-expressing the enzyme and taken up by deficient cells, which makes TPP I an attractive candidate for the development of gene-based therapies. In the experiments of Lin and Lobel [16], Chinese-hamster ovary cells (CHO) were transfected with human CLN2 and selected to secrete high levels of the enzyme. The enzyme was delivered *in vitro* to the lysosomes of LINCL fibroblasts by mannose-6-phosphate receptor mediated endocytosis, which restored the normal enzyme activity levels and preserved the accumulation of the auto-fluorescent material in the lysosomes. The recent development of a mouse-model of LINCL has greatly accelerated and simplified the studies, related with the gene-therapy application [27]. Passini et al. [23] have shown that CLN2 (-/-) mice, injected into the brains by adeno-associated virus vectors containing the human CLN2 cDNA had a marked reduction of auto-fluorescent storage material in the cells throughout the CNS. Thus, the gene-replacement therapy corrects the cellular pathologies of LINCL in the mouse model and raises the possibility of using gene therapy to treat LINCL patients.

TPP I as a marker for different diseases

Aberrant TPP I expression has been found in different diseases. For example, increased TPP I levels have been detected in aging brain, neurodegenerative diseases [10], lysosomal storage disorders, and some differentiated neoplasms, whereas decreased levels of the enzyme have been reported in ischemic/anoxic areas and undifferentiated tumors (for review see [14]). Tripeptidyl-peptidase I showed a significant increase in squamous cell carcinomas of the lower third of the esophagus as compared to the levels of activity measured in the bordering intact mucosa [2]. Junaid et al. [13] have measured TPP I levels in breast tissue samples from normal subjects undergoing reductive mammoplasty and patients with primary breast carcinoma. The results showed a two- to seventeen-fold higher CLN2 protein activity in tumours, which was significantly and positively correlated with already known breast cancer biomarkers such as levels of cathepsin D, estrogen receptor and progesterone receptor. These results suggest a diagnostic and prognostic potential for TPP I in breast cancer.

Methods for the *in situ* studies of TPP I expression

The in situ investigations on the enzyme are now performed exclusively by immuno-cyto- and immunohistochemical methods [15, 35]. Previously, we developed a novel chromogenic substrate for TPP I – Gly-Pro-Met-1-anthraquinonyl hydrazide, which

was intended for the light microscopy histochemical study of the enzyme [4]. Based on the histochemical principle, proposed by us, Davidson and Viemer [3] invented a patent, in which the compound Gly-Pro-Ser-1-anthraquinonyl hydrazide was synthesized and used for the visualization of TPP I in rat kidney. However, neither our studies nor the comprehensive investigations of Tlan et al. [30] on the enzyme specificity have ever proved that serine at P1 position is better than methionine as substrate for TPP I.

Fluorogenic methods are much more sensitive and selective than chromogenic methods for the visualization of enzyme activities. The availability of a sensitive and selective cyto- and histochemical fluorogenic assay of TPP I would give the possibility not only to study thoroughly the distribution of the enzyme throughout the mammalian organs and tissues, but also to diagnose CLN2 deficiency by a simple enzyme activity-based test, to evaluate the effectiveness of gene therapy *in situ* and to elucidate the role of the enzyme in the development and progression of the diseases, for which it is proposed as a marker. Previously, we introduced a fluorescent histochemical procedure for the visualization of TPP I activity, based on 2-anthraquinonyl hydrazide substrates and 3-nitrobenzaldehyde as visualization agent [5]. Due to the very difficult synthetic procedure, which uses cancerogenic compounds and the application of 3-nitrobenzaldehyde, which binds to the free amino group of the substrate, thus decreasing the substrate access in the incubation medium, this procedure was not accepted for routine applications. Presently, we developed novel TPP I substrates – 4-Gly-Pro-Met-hydrazydo-N-hexyl-1,8-naphthalimide and 4-Ala-Ala-Phe-hydrazydo-N-hexyl-1,8-naphthalimide, which are convenient for the fluorescent histochemical studies on TPP I activity [9]. The principle of the novel technique is shown on Fig.1. The enzyme cleaves the hydrazide bond at the carboxyl group of methionine or phenylalanine to re-

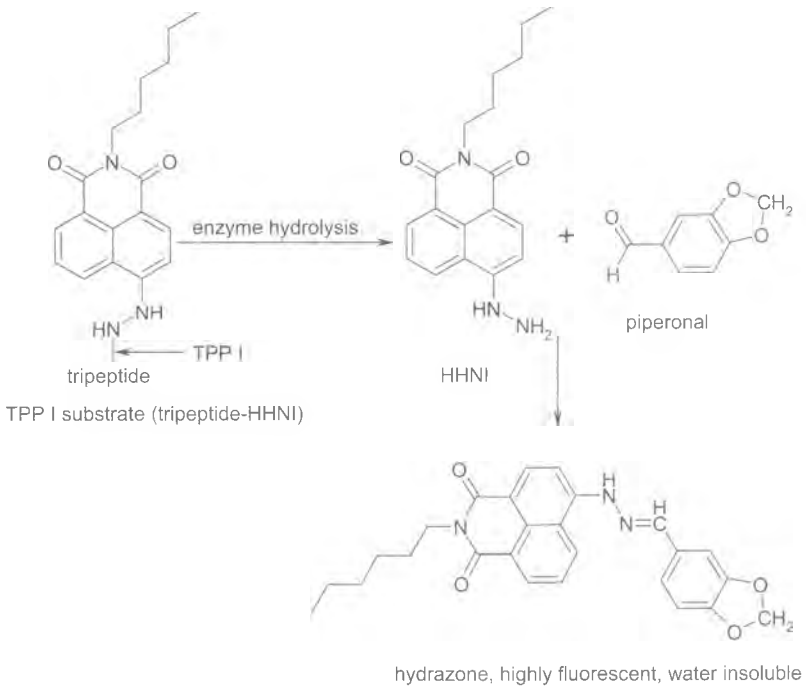

Fig 1. Histochemical principle to visualize TPP I activity.

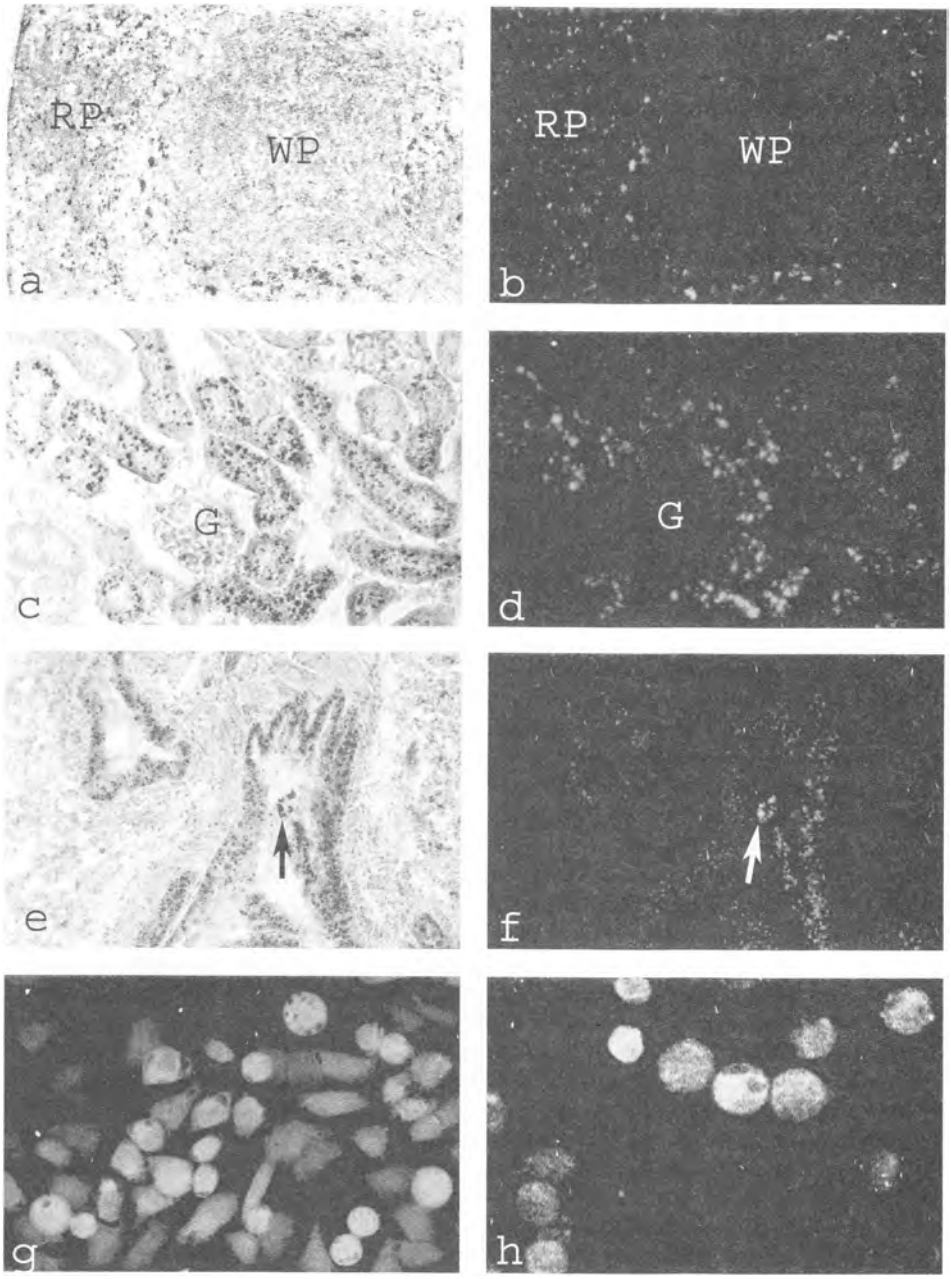

Fig. 2. Localization of TPP I activity. a-f: tissue sections of Wistar rat organs using the substrate Gly-Pro-Met-4-hydrazido-N-hexyl-1,8-naphthalimide and piperonal as auxiliary reagent (a, c, e, - light microscopy; b, d, f – fluorescent microscopy). a, b – spleen: reaction product in individual cells within the red pulp (RP); no reaction in white pulp (WP), x 200; c, d – kidney: convoluted tubules are highly TPP I – positive, whereas the glomeruli (G) are negative, x 200; e, f – lung: enzyme reaction in the epithelial cells of the bronchi and bronchial macrophages (arrow), x 200; g, h – TPP I-positive human HepG2 cells (hepatocellular carcinoma) – substrate Ala-Ala-Phe-4-hydrazido-N-hexyl-1,8-naphthalimide, visualization agent – piperonal; g: x 500; h: x 750.

lease the compound 4-hydrazyno-N-hexyl-1,8-naphthalimide, which reacts quickly and quantitatively with an aromatic aldehyde (piperonal) to obtain a highly water-insoluble hydrazone. The last compound precipitates on the sites of enzyme activity and marks them by a stable red fluorescence. Using the novel substrates we were able to visualize the enzyme activity in tissue sections of different rat and mice organs and in human tumor cells. The substrates are easier to synthesize using established chemical methods and the synthetic process does not involve dangerous compounds. Another advantage of the novel procedure is that the used aldehyde does not react with the free amino group of the substrate and thus, the substrate remains available in the solution throughout the whole incubation process. Enzyme locations can be observed both by light and fluorescent microscopy. Examples of visualization of TPP I activity by this principle are shown on Fig. 2. The novel fluorescent technique might open new possibilities in the study of TPP I activity in normal and pathologically altered tissues and may be useful for the diagnosis of LINCLE.

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References

1. Букина, А. М., И. В. Цветкова, А. Н. Семячкина, Е. С. Ильина. Недостаточность трипептидил пептидазы I при нейрональном цероидном липофусцинозе. Новая мутация. – Вопр. Мед. Химии, **48,** 2002, 594-598
2. Altorjay, A., B. Paal, N. Sohar, J. Kiss, I. Szanto, I. Sohar. Significance and prognostic value of lysosomal enzyme activities measured in surgically operated denocarcinomas of the gastroesophageal junction and squamous cell carcinomas of the lower third of esophagus. – World J Gastroenterol., **11**, 2005, 5751-5756.
3. Davidson, B. L., D. Wiemer. Compounds and methods for detecting tripeptidyl peptidase I. – US Patent 6,824,998 B2, 2004.
4. Dikov, A., M. Dimitrova, I. Ivanov, R. Krieg, K.-J. Halbhuber. Original method for the histochemical demonstration of tripeptidyl aminopeptidase I. – Cell. Mol. Biol., **46**, 2000, 1219-1226.
5. Dikov, A., M. Dimitrova, R. Krieg, K.-J. Halbhuber. New fluorescent method for the histochemical detection of tripeptidyl peptidase I using glycyl-L-prolyl-L-met-2-anthraquinonyl hydrazide as substrate. – Cell. Mol. Biol., **50,** 2004, 565-568.
6. Du, P-G., S. Kato, Y-H. Li, T. Maeda, T. Yamane, S. Yamamoto, M. Fujiwara, Y. Yamamoto, K. Nishi, I. Ohkubo. Rat tripeptidyl peptidase I: molecular cloning, functional expression, tissue localization and enzymatic characterization. – Biol. Chem., 382, 2001, 1715-1725.
7. Golabek, A., E. Kida, M. Walus, P. Wujek, P. Mehta, K. Wisniewski. Biosynthesis, glycosylation and enzymatic processing in vivo of human tripeptidyl peptidase I. – J. Biol. Chem., 278, 2003, 7135-7145.
8. Golabek, A., P. Wujek, M. Walus, S. Bieler, C. Soto, K. Wisniewski, E. Kida. Maturation of human tripeptidyl peptidase I in vitro. – J. Biol. Chem., 279, 2004, 31058-31067.
9. Ivanov, I., D. Tasheva, R. Todorova, Dimitrova. Synthesis and use of 4-peptidyl hydrazido-N-hexyl-1,8-naphthalimides as fluorogenic histochemical substrates for dipeptidyl peptidase IV and tripeptidyl peptidase I. – Eur. J. Med. Chem., **44**, 2009, 384-392.
10. Junaid, M. A., R. K. Pullarkat. Increased brain lysosomal pepstatin-insensitive proteinase activity in patients with neurodegenerative diseases. – Neurosci. Lett., 264, 1999, 157-160.
11. Junaid, M. A., S. S. Brooks, K. E. Wisniewski, R. K. Pullarkat. A novel assay for lysosomal pepstatin-insensitive proteinase and its application for the diagnosis of late-infantile neuronal ceroid lipofuscinosis. – Clin. Chim. Acta, 281, 1999,169-176.

12. Junaid, M. A., G. Wu, R. K. Pullarkat. Purification and characterization of bovine brain lysosomal pepstatin-insensitive proteinase, the gene product deficient in the human late-infantile neuronal ceroid lipofuscinosis. – J. Neurochem. 74, 2000a, 287-294.
13. Junaid, M. A., G. M. Clark, R. K. Pullarkat. A lysosomal pepstatin-insensitive proteinase as a novel biomarker for breast carcinoma. – Int. J. Biol. Markers, 15, 2000b, 129-134.
14. Kida, E., A. A. Golabek, M. Walus, P. Wujek, W. Kaczmarski, K. E. Wisniewski. Distribution of tripeptidyl peptidase I in human tissues under normal and pathological conditions. – J. Neuropathol. Exp. Neurol., 60, 2001, 280-292.
15. Koike, M., M. Shibata, Y. Ohsawa, S. Kametaka, S. Waguri, E. Kominami, Y. Uchiyama. The expression of tripeptidyl peptidase I in various tissues of rats and mice. – Arch. Histol. Cytol., 65, 2002, 219-232.
16. Lin, L., P Lobel. Production and characterization of recombinant human CLN2 protein for enzyme-replacement therapy in late infantile neuronal ceroid lipofuscinosis. – Biochem J., 357, 2001, 49–55.
17. Lin, C. G., D. E. Sleat, R. J. Donnelly, P. Lobel. Structural organization and sequence of CLN2, the defective gene in classical late infantile neuronal ceroid lypofuscinosis. – Genomics, 50, 1998, 206-212.
18. Lin, L., I. Sohar, H. Lackland, P Lobel. The human CLN2 protein/tripeptidyl-peptidase I is a serine protease that autoactivates at acidic pH. – J. Biol. Chem. 276, 2001, 2249–2255.
19. McDonald, J. K., A. R. Hoisington, D. A. Eisenhauer. Partial purification and characterization of an ovarian tripeptidyl peptidase: a lysosomal exopeptidase that sequentially releases collagen-related (Gly-Pro-X) triplets. – Biochem. Biophys. Res. Commun. 126, 1985, 63-71.
20. Mole, S. E., R. E. Williams, H. H. Goebel. Correlations between genotype, ultrastructural morphology and clinical phenotype in the neuronal ceroid lipofuscinoses. – Neurogenetics, 6, 2005,107-126.
21. Page, A. E., K. Fuller, T. J. Chambers, M. J. Warburton. Purification and characterization of a tripeptidyl peptidase I from human osteoclastomas: evidence for its role in bone resorption. – Arch. Biochem. Biophys, 307, 1993, 354-359.
22. Palmer, D. N, I. M. Fearnley, J. E. Walker, N. A. Hall, B. D Lake, L. S. Wolfe, M. Haltia, R. D. Martinus, R. D. Jolly. Mitochondrial ATP synthase subunit c storage in the ceroid-lipofuscinoses (Batten disease). – Am. J. Med. Genet., 42, 1992, 561-567.
23. Passini, M. A., J. C. Dodge, J. Bu, W. Yang, Q. Zhao, D. Sondhi, N. R. Hackett, S. M. Kaminsky, Q. Mao, L. S. Shihabuddin, S. H. Cheng, D. E. Sleat, G. R. Stewart, B. L. Davidson, P. Lobel, R. G. Crystal. Intracranial delivery of CLN2 reduces brain pathology in a mouse model of classical late infantile neuronal ceroid lipofuscinosis. – J. Neurosci., 26, 2006, 1334-1342.
24. Rawlings, N. D., A. J. Barett. Tripeptidyl peptidase I is appearently the CLN2 protein absent in classical late-infantile neuronal ceroid lipofuscinosis. – Biochim. Biophys. Acta, 1429, 1999, 496-500.
25. Sleat, D. E., R. J. Donnelly, H. Lackland, C. G. Lin, I. Sohar, R. K. Pullarkat, P. Lobel. Association of mutations in a lysosomal protein with classical late-infantile neuronal ceroid lipofuscinosis. – Science, 277, 1997, 1802-1805.
26. Sleat, D. E, R. M. Gin, I. Sohar, K. Wisniewski, S. Sklower-Brooks, R. K. Pullarkat, D. N. Palmer, T. J. Lemer, R. – M. Boustany, P. Uldall, A. N. Siakotos, R. Donnelley, P. Lobel. Mutational analysis of the defective protease in classical late-infantile neuronal ceroid lypofuscinosis, a neurodegenerative lysosomal storage disorder. – Am. J. Hum. Genet., 64,1999, 1511-1523.
27. Sleat, D. E.,J. A. Wiseman, M. El-Banna,. K. H. Kim, Q. Mao, S. Price, S. L. Macauley, R. L. Sidman, M. M. Shen, Q. Zhao, M. A. Passini, B. L. Davidson, G. R. Stewart, P. Lobel. A mouse model of classical late-infantile neuronal ceroid lipofuscinosis

based on targeted disruption of the CLN2 gene results in a loss of tripeptidyl-peptidase I activity and progressive neurodegeneration. – J. Neurosci. 2, 2004, 9117-9126.
28. Sohar, I., D. E. Sleat, M. Jadot, P. Lobel. Biochemical characterization of a lysosomal protease deficient in classical late infantile neuronal ceroid lipofuscinosis (LINCL) and development of an enzyme-based assay for diagnosos and exclusion of LINCL in human specimens and animal models. – J. Neurochem., 73, 1999, 700-711.
29. Sohar, I., L. Lin, P. Lobel. Enzyme-based diagnosis of classical late-infantile neuronal ceroid lypofuscinosis: compareson of TPP I and pepstatin-insensitive protease assays. – Clin. Chem., 46, 2000, 1005-1008.
30. Tlan, Y., I. Sohar, J. Taylor, P. Lobel. Determination of substrate specificity of tripeptidyl peptidase I using combinatorial peptide libraries and development of improved fluorogenic substrates. – J. Biol. Chem., 281, 2006, 6559: 65-72.
31. Vines, D. M. J. Warburton. Purification and characterization of a tripeptidyl peptidase I from rat spleen. – Biochim. Biophys. Acta, 1384, 1998, 233–242
32. Vines, D. J., M. J. Warburton. Classical late-infantile neuronal ceroid lypofiscinosis fibroblasts are deficient in lysosomal tripeptidyl peptidase I. – FEBS Lett., 443, 1999, 131-135.
33. Wisniewski, K. E., A. Kaczmarski, E. Kida, F. Connell, W. Kaczmarski, M. P. Michalewski, D. N. Moroziewicz, N. Zhong, A. M. Das R. D. Jolly, A. Kohl-Schutter. Reevaluation of neuronal ceroid lipofuscinosis: atypical juvenile onset may be the result of CLN2 mutations. – Mol. Genet. Metabol., 66, 1999, 248-252.
34. Wlodawer, A, M. Li, Z. Dauter, A. Gustchina, K. Uchida, H. Oyama, B. M. Dunn, K. Oda. Carboxyl proteinase from Pseudomonas defines a novel family of subtilisin-like enzymes. – Nat. Struct. Biol., 8, 2001, 442-446.
35. Yayoi, Y., Y. Ohsawa, M. Koike, G. Zhang, E. Kominami, Y. Uchiyama. Specific localization of lysosomal aminopeptidases in type II alveolar epithelial cells of the rat lung. – Arch. Histol. Cytol., 64, 2001, 89-97.