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Multiple γ-glutamylation: A novel type of post-translational modification in a diapausing *Artemia* cyst protein

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ABSTRACT

A highly hydrophilic, glutamate-rich protein was identified in the aqueous phenol extract from the cytosolic fraction of brine shrimp (*Artemia franciscana*) diapausing cysts and termed *Artemia* phenol soluble protein (PSP). Mass spectrometric analysis revealed the presence of many protein peaks around *m/z* 11,000, separated by 129 atomic mass units; this value corresponds to that of glutamate, which is strongly suggestive of heterogeneous polyglutamylation. Polyglutamylation has long been known as the functionally important post-translational modification of tubulins, which carry poly(L-glutamic acid) chains of heterogeneous length branching off from the main chain at the γ-carboxy groups of a few specific glutamate residues. In *Artemia* PSP, however, Edman degradation of enzymatic peptides revealed that at least 13, and presumably 16, glutamate residues were modified by the attachment of a single L-glutamate, representing a hitherto undescribed type of post-translational modification: namely, multiple γ-glutamylation or the addition of a large number of glutamate residues along the polypeptide chain. Although biological significance of PSP and its modification is yet to be established, suppression of *in vitro* thermal aggregation of lactate dehydrogenase by glutamylated PSP was observed.

Introduction

Post-translational modification (PTM) of protein is probably the most important regulatory mechanism of protein function. A typical PTM is phosphorylation, which reversibly introduces a highly acidic phosphoryl group to the modified amino acid and is central to signal transduction in numerous cellular processes. Usually PTMs are restricted to a single or small numbers of critical amino acid residues and bring about a minute, but functionally significant, local or overall conformational rearrangement to the modified protein [1]. A single protein can be simultaneously modified with different kinds of PTM; an example of which is histone modification by acetylation, methylation, phosphorylation and ubiquitination [1]. In rare instances, a large number of residues undergo a single PTM within a molecule; for example, RNA polymerase II bears many phosphoryl groups on about 50 tandemly repeated heptapeptide sequences in the C-terminal domain [2] and Antarctic fish anti-freeze glycoproteins are comprised of up to 50 repeating Ala-Ala-Thr tripeptide units where every threonine residue is glycosylated with the disaccharide β-D-galactosyl-(1→3)-α-*N*-acetyl-D-galactosamine [3]. Blood coagulation factors and cartilage proteins also contains many γ-carboxyglutamates [4,5]. These extensive covalent modifications should lead to profound changes in physicochemical, and therefore functional, properties of the original protein molecule. Here, we report the identification of a novel type of extensive PTM: namely, the addition of glutamate to many glutamate residues in a protein of diapausing *Artemia* cysts, which are renowned for their extraordinary tolerance to various environmental stresses [6].

Material and methods

Materials. Artemia franciscana cysts were obtained from aQua Corporation (Osaka, Japan) and had been collected at Great Salt Lake. Synthetic γ-glutamylglutamic acid (γ-Glu-Glu) was a product of MP Biomedicals, Inc. (Solon, Ohio, US).

Abbreviations: PSP, phenol soluble protein; PTM, post-translational modification; LEA, late embryogenesis abundant; MALDI-TOF-MS, matrix-associated laser-desorption-ionization time-of-flight mass spectrometry; PTH, phenylthiohydantoin; RACE, rapid amplification of cDNA ends; LDH, lactate dehydrogenase; BSA, bovine serum albumin; ESI-IT-TOF-MS, electrospray-ionization ion-trap time-of-flight mass spectrometry.

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Isolation of Artemia PSP from cysts. Cysts (60 g) were treated with ice cold 1% sodium hypochlorite for 5 min, and washed first four times with 1 l each of cold water, and then with 300 ml of cold buffer A (35 mM Tris–HCl/1 mM $MgCl_2$/70 mM KCl/0.1 mM EDTA/0.25 M sucrose/10 mM 2-mercaptoethanol). The cytosol fraction was prepared by grinding cysts in a mortar grinder with 300 ml of buffer A and centrifugation at 200,000*g* for 90 min. All operations were carried out at below 4 °C. The cytosol fraction was diluted with an equal amount of water, mixed with a half volume of Tris-saturated phenol, and centrifuged (5000*g*) for 10 min to separate the aqueous layer. The aqueous layer was stirred with an equal volume of chloroform and centrifuged to remove residual phenol. To this were added 0.1 part of 3 M sodium acetate and 2.5 parts of ethanol, and the solution was allowed to stand for 30 min at −20 °C. The precipitate formed was collected by centrifugation and washed with 70% ethanol. The bulk of RNA present was removed by digestion with RNase A (5 μg) for 1 h at 37 °C and extensive dialysis. The protein was purified by reversed-phase HPLC on a CapCell PAK C8 column (SHISEIDO, Tokyo, Japan) by using a linear gradient of acetonitrile concentration in 0.05% trifluoroacetic acid.

Amino acid and sequence analysis, mass spectrometry cDNA cloning and expression of recombinant protein are described in Supplementary material.

Identification of the modified glutamate residue by protein sequencer. The phenylthiohydantoin (PTH) derivative of the modified glutamate residue, which eluted around the flow-through position by HPLC on the ABI protein sequencer, could be identified by prior methylation of peptides. Peptides (1 nmol) were dried by lyophilization and methylated in 50 μl of 3 N methanolic HCl for 90 min at 50 °C. Methylated peptides were lyophilized in a vacuum concentrator, and dissolved in 50% acetonitrile for sequencing. By this method, both modified and unmodified glutamate were identified as double peaks due to partial hydrolysis of γ-methyl ester during the conversion step in 25% aqueous trifluoroacetic acid at 64 °C. This finding was confirmed by manually collecting the PTH derivatives at the injection port of HPLC system in the protein sequencer. The derivatives were dehydrated, methylated again by methanolic HCl and then analyzed with the built-in HPLC systems. The release of methyl groups in peptides during repeated sequencing reactions was negligible.

Protection against thermal aggregation of protein. Turbidimetric measurements of protein denaturation and aggregation were made on heated reaction mixtures containing lactate dehydrogenase (LDH, rabbit muscle, Sigma) at 5 μM (subunit concentration) and *Artemia* protein at 0.5, 1.0, 2.5, and 5 μM in 40 mM Hepes/KOH buffer (pH 7.5) [7]. Bovine serum albumin (BSA, Sigma) was used to examine non-specific protection. Protein solutions were filtered with 0.22-μm membrane filters (Millipore) before use, and concentrations were determined by absorbance at 280 nm (LDH and BSA) or by amino acid analysis (*Artemia* protein). Turbidity was measured at 340 nm in a cuvette maintained at 43 °C with a Hitachi 220S spectrophotometer. All assays were done in triplicate.

Results

Isolation of Artemia PSP from cysts

When the ethanol precipitate of the phenolic extract of the cytosol fraction was subjected to electrophoresis on a 6 M urea/polyacrylamide gel and stained with Azur B, a classical stain for RNA, a dense band was seen other than those of tRNAs (Fig. 1A). A sufficient amount of this protein for further analyses was obtained by reversed-phase HPLC after the removal of bulk RNA by RNase A digestion, followed by dialysis (Fig. 1B). The protein, termed *Artemia* phenol soluble protein (PSP), was stained by Coomassie Brilliant Blue as an 18-kDa band on SDS–PAGE (Fig. 1C). Table 1 shows its characteristic amino acid composition, which comprises an unusually high content of hydrophilic amino acids, including over 50 mol% of glutamate, and a very low content of hydrophobic residues (15 mol%), lacking the typical hydrophobic residues, Leu, Ile, Tyr and Trp. The isoelectric point was calculated as 3.83; this acidic nature of PSP may account for its slow migration on SDS–PAGE due to lesser SDS binding. About 2 mg protein was purified from 60 g of dry cysts.

Sequence analysis

Direct sequence analysis of the protein indicated the presence of a blocked N-terminus. Assuming a prevailing modification of

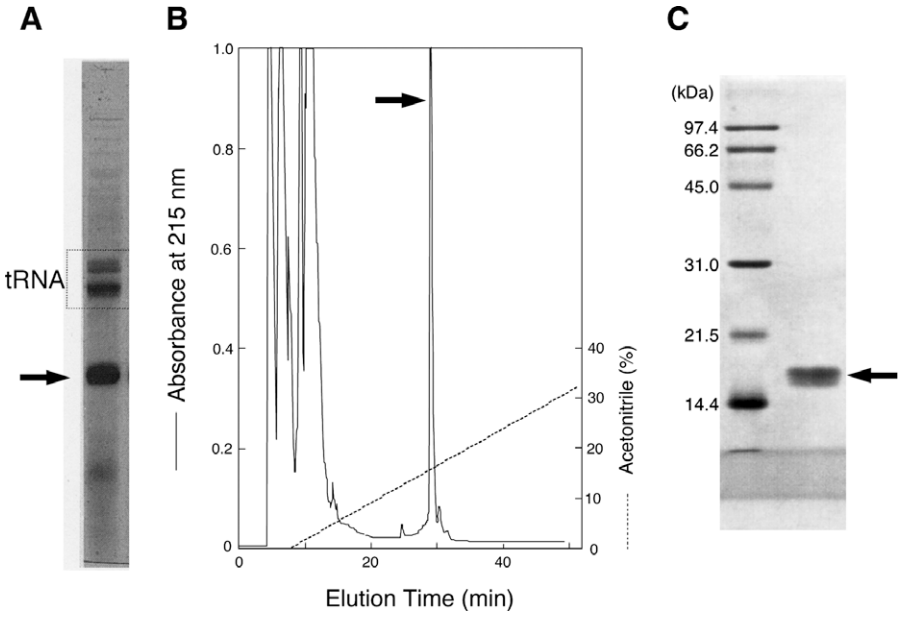

Fig. 1. Detection and purification of *Artemia* phenol soluble protein (PSP). (A) Electrophoresis of cytosolic RNA (10 μg) on a 6 M urea–polyacrylamide gel stained by RNA stain Azur B. Arrow indicates an unknown band stained by Azur B. (B) Reversed-phase HPLC of the cytosolic RNA fraction digested with RNase A. The bulk of the RNA digest eluted at the flow-through position, and a single peak of PSP was obtained (arrow). (C) SDS–polyacrylamide gel electrophoresis of PSP (5 μg) obtained from HPLC and stained by Coomassie Brilliant Blue. Standard proteins were separated in the leftmost lane. PSP (arrow) is very acidic and appears to show an anomalously low electrophoretic mobility due to lesser SDS binding.

Table 1
Amino acid composition of *Artemia* cyst phenol soluble protein (PSP) and its recombinant protein expressed in *E. coli*.

Amino acid	*Artemia* PSP		
	Cyst-derived	cDNA	Recombinant
Asp	7.4 (7)	7	7.0 (7)
Thr	4.8 (5)	5	4.6 (5)
Ser	0.9 (1)	1	1.7* (2)
Glu	53.2 (53)	39	39.2 (39)
Pro	1.9 (2)	2	3.1* (3)
Gly	10.4 (10)	10	12.0* (12)
Ala	5.8 (6)	6	6.1 (6)
Cys	0 (0)	0	0 (0)
Val	6.5 (7)	7	6.6 (7)
Met	0 (0)	0	1.3* (1)
Ile	0 (0)	0	0 (0)
Leu	0 (0)	0	1.3* (1)
Tyr	0 (0)	0	0 (0)
Phe	1.1 (1)	1	0.9 (1)
Lys	9.4 (9)	9	9.0 (9)
His	0 (0)	0	0 (0)
Trp	0 (0)	0	0 (9)
Arg	1.0 (1)	1	0.8 (1)
Total	102	89	93

Numbers in parentheses are the nearest integers. Asn and Gln are counted as Asp and Glu, respectively. Asterisks (*) indicate the inclusion of extra amino acids derived from a short segment in the linker region of the recombinant fusion protein.

acetylation, we successfully removed the blocking acetyl group by methanolysis [8], and determined the N-terminal 36-amino acid sequence. Because *Artemia* PSP contains a single arginine residue, it was digested with arginylendopeptidase (Arg-C) and 17 residues were directly analyzed. Results of these sequence analyses yielded a contiguous 40-amino acid sequence from the N-terminus (summarized in Supplementary Fig. S1).

cDNA-cloning of Artemia PSP

The nucleotide sequence of the N-terminal region was first determined by using two sets of degenerate primers derived from the N-terminal amino acid sequence. Subsequently, using primers based on the N-terminal nucleotide sequence, the whole coding sequence was determined by 5′- and 3′-RACE. These sequencing results are shown in Supplementary Fig. S1, and amino acid composition calculated from the deduced primary structure is shown in Table 1. As can be seen from Table 1, the result of the amino acid analysis of the cyst protein is consistent with that expected from the cDNA sequence, except for the content of glutamate. The value for glutamate content in the cyst protein is about 20% larger than the theoretical value. The origin of this significant discrepancy was analyzed by mass spectrometry and chemical analysis as described below.

Mass spectrometric analysis

MALDI-TOF-MS of PSP revealed at least 13 peaks, differing from each other by 129–130 amu (Fig. 2). Analysis by ESI-IT-TOF-MS (electrospray-ionization ion-trap time-of-flight mass spectrometry) gave more accurate values for the molecular mass of the protein, although the number of interpretable peaks was reduced to four (Supplementary Fig. S2). The mass values obtained by deconvolution of the multivalent protein peaks were 10,996.3181, 11,125.5993, 11,254.5859 and 11,383.6332; the average mass difference between neighboring peaks was 129.105, a value corresponding to the mass of glutamic acid (Fig. S2). The deviations of these four mass values from the theoretical one of acetylated, unmodified protein (9962.9) were 1033.4, 1162.7, 1291.7 and 1420.7, which can be interpreted as $129n$ ($n = 8$ to 11). From the 13 peaks visible by MALDI-TOF-MS (Fig. 2), the number of added glutamate residues may range from 4 to 16 residues. This result suggested the post-translational addition of short poly(glutamic acid) chains of 4–16 glutamate residues, known as polyglutamylation, first discovered in α-tubulin in 1990 [9].

Identification of the modified residues

Assuming the presence of polyglutamylation, we analyzed the site of modification by peptide analysis. Because an initial sequence analysis did not identify an unusual amino acid in the first 40 amino acids from the N-terminus, the modified site(s) was ex-

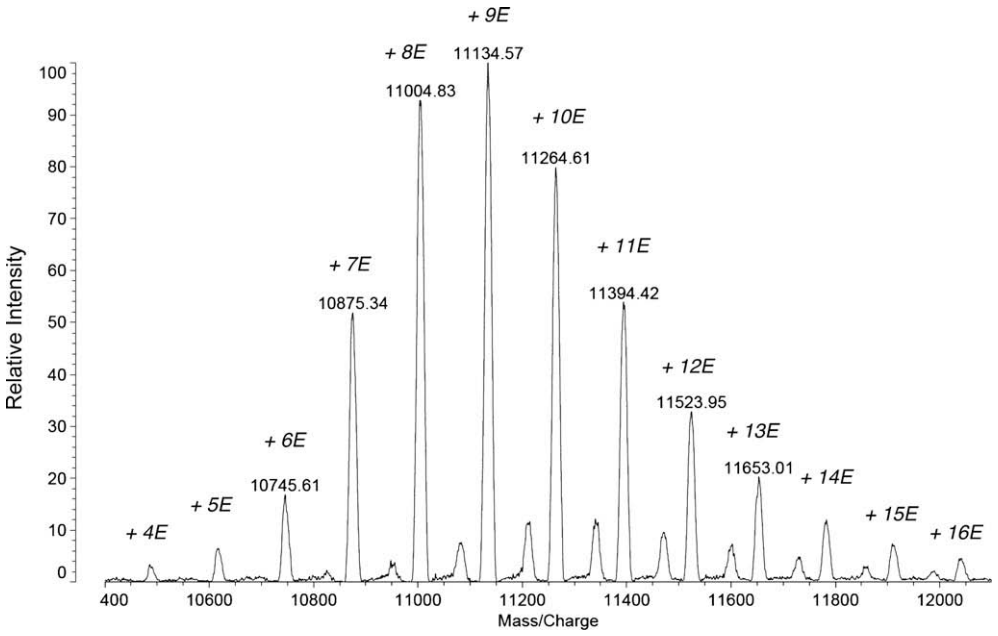

Fig. 2. Mass spectrum of *Artemia* PSP by MALDI-TOF-MS. Data were collected in linear mode at an extraction potential of 20 kV in positive-ion mode. The numbers shown on each protein peak are the observed molecular mass and estimated numbers of extra glutamates (E). The origin of the accompanying small peaks is unknown.

Table 2
Amino acid composition and molecular mass of the thermolysin peptides Th2a–Th2e.

Amino acid	Theory	Thermolysin peptides Th2a–Th2e				
		Th2a	Th2b	Th2c	Th2d	Th2e
Asp	2	2.0 (2)	2.0 (2)	2.0 (2)	2.0 (2)	1.9 (2)
Glu	14	16.6 (17)	17.6 (18)	19.2 (19)	20.2 (20)	21.1 (21)
Gly	5	5.0 (5)	5.2 (5)	5.2 (5)	5.2 (5)	5.2 (5)
Ala	2	1.7 (2)	1.8 (2)	1.7 (2)	1.8 (2)	1.7 (2)
Phe	1	1.1 (1)	1.0 (1)	1.2 (1)	0.9 (1)	0.9 (1)
Total	24	27	28	29	30	31
Mass: observed[a]		3015.01	3144.17	3273.15	3402.30	3531.38
Mass: theory	2628.91	+3 Glu	+4 Glu	+5 Glu	+6 Glu	+7 Glu
		3016.04	3145.08	3274.12	3403.17	3532.21

Numbers in parentheses are the nearest integers.
[a] Observed in negative-ion mode, $[M-H]^-$.

pected to be in the C-terminal half of the protein. Therefore, PSP was first digested with lysylendopeptidase (Lys-C) and the peptides were separated by reversed-phase HPLC (Supplementary Fig. S3A) to obtain a large C-terminal peptide, Lys-C3, for structural analysis. This peptide was further digested with thermolysin, and the resultant peptides were separated by reversed-phase HPLC. The elution profile in Supplementary Fig. S3B shows a set of peptides eluting closely together (Th2a–Th2e) and several other peptides (Th1, Th3 and Th4).

The peptides Th2a–Th2e were further purified by reversed-phase HPLC, and analyzed for amino acid composition and molecular mass. The results are summarized in Table 2. The peptides were obviously derived from the C-terminal quarter of the protein, from Phe-66 to Ala-89, because the sole phenylalanine in the protein was present. However, an additional three to seven residues of glutamate were found in these peptides. This finding was consistent with the results of MALDI-TOF-MS in negative-ion mode (Table 2). Sequence analysis of Th-2d, which had an additional six glutamates, gave the corresponding C-terminal sequence, FDEEA EGEDEEGEGE GEGEEEEEA (residue 66–89), but six positions (underlined in the sequence) where glutamate was expected were blank, and instead a putative 'nonstandard' PTH-amino acid was found slightly before PTH-aspartic acid (Supplementary Fig. S4B). This observation led us to hypothesize that the modified residues were γ-glutamylglutamic acid (γ-Glu-Glu or γ-E-E), because six glutamate residues were modified in Th-2d, which had six extra glutamates.

The PTH derivative of authentic γ-Glu-Glu also eluted at the same position. Because this position overlapped with that of oxidized dithiothreitol contained in the sequencer reagents, unambiguous identification of modified glutamic acid was necessary. To achieve this, the peptide samples were first methylated by methanolic HCl, which was expected, and indeed observed, to increase the hydrophobicity of the modified residue for retention on the reversed-phase column. However, the protein sequencer utilizes 25% aqueous trifluoroacetic acid at 64 °C in the final conversion step, which resulted in partial hydrolysis of the methyl esters, yielding two peaks of di- and mono-methyl esters for PTH-γ-Glu-Glu (Supplementary Fig. S4C). Unmodified glutamic acid also gave dual peaks corresponding to PTH-glutamic acid γ-methyl ester and PTH-glutamic acid (e.g. at cycle 11 in Fig. S5). This interpretation was verified by sequencer analysis of synthetic γ-Glu-Glu (Supplementary Fig. S4E) or standard glutamic acid (Supplementary Fig. S4G) methylated by methanolic HCl. To further confirm the nature of the double peak, PTH derivatives were collected at the injection port of the sequencer HPLC, remethylated with methanolic HCl and analyzed. The PTH derivatives from methylated peptide (Th2e, Supplementary Fig. S4D) and authentic compounds (Supplementary Fig. S4F, H) converged into single peaks. Therefore, the double peak observed in sequence analysis of the methylated peptides was concluded to be unambiguous proof for the modified glutamic acid. Aspartic acid β-methyl ester was rather stable and identified mainly as a single peak around PTH-Ala (for example, Supplementary Fig. S5, cycle 2).

Supplementary Fig. S5 shows a typical result of sequence analysis of a methylated peptide, Th2e, with seven extra glutamates. Six positions were identified as γ-Glu-Glu (denoted as γ-E-E in Fig. S5) and, at the 6th and 17th positions, equal amounts of γ-Glu-Glu and Glu (E) were detected. This observation indicated that Th2e contained two peptides with the same number of extra glutamic acids (seven residues); six of these extra residues were on the same glutamate residues in the two peptides, but the 7th extra glutamic acid was located on different positions. The positions of γ-glutamylation in Th2a–Th2e are summarized in Supplementary Fig. S6.

The other thermolysin-derived peptides located in the N-terminal half of peptide LysC-3 from Th2 were rather homogeneous with respect to γ-glutamylation, and the five modified positions were identified by peptide sequencing. An absence of modified glutamate residues in the N-terminal half of the protein was suggested by initial sequence analysis of the deblocked protein and verified by analysis of a Lys-C peptide, Lys-C1, and an endoproteinase Asp-N peptide, Asp-N4, which was generated by unusual hydrolysis at the N-terminal side of Glu-4 by Asp-N protease. Direct sequence analysis of Lys-C3 also confirmed the absence of modification at Glu-39, Glu-40 and Glu-42. These sequence analyses were carried out on methylated peptides. The overall structure of *Artemia* PSP with potential 13 γ-glutamylation sites is presented in Fig. 3. The configuration of the attached glutamic acids was examined in the thermolysin peptide Th2e by derivatization with Marfey's reagent [10], and it was identified as the usual L-enantiomer.

Preparation of recombinant Artemia PSP

Amino acid analysis of the recombinant PSP protein (Table 1) showed the presence of the same number of glutamate residues as expected from the cDNA sequence, indicating that the glutamylation reaction did not take place in the *Escherichia coli* system.

Aggregation-protecting activity of native and recombinant proteins

The highly hydrophilic nature of *Artemia* PSP and its presence in dormant cysts was reminiscent of the plant seed stress proteins, LEA (late embryogenesis abundant) proteins, that protect seeds from various environmental stresses and are also very hydrophilic [reviewed in 11–13]. We therefore examined whether the *Artemia* PSP protein can also protect proteins from thermal denaturation and aggregation by using rabbit muscle lactate dehydrogenase (LDH) as a model protein [7,14]. Fig. 4A shows the kinetics of

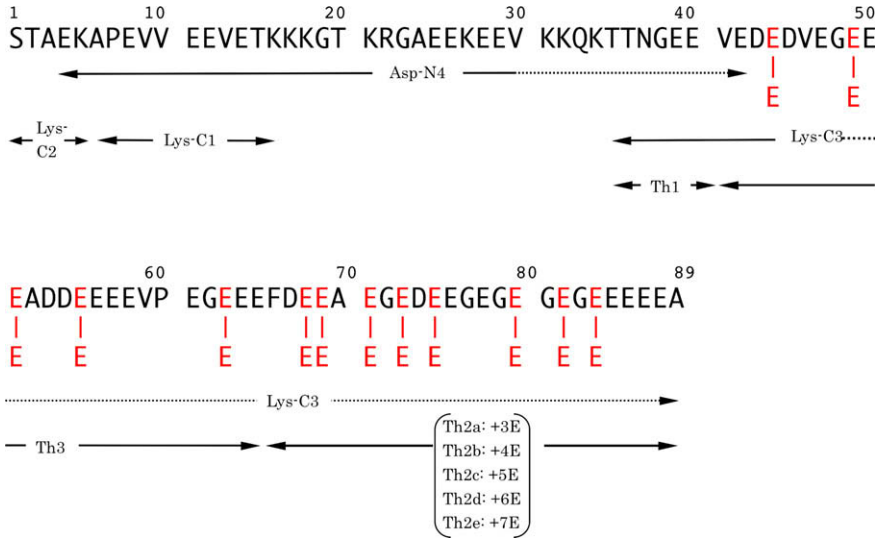

Fig. 3. Amino acid sequence of *Artemia* PSP with sites of post-translational γ-glutamylation. Enzymatic peptides generated by a lysylendopeptidase, thermolysin, and endopeptidase Asp-N digestion are denoted by Lys-C, Th, and Asp-N, respectively. Solid lines indicate the sequence experimentally determined, and dotted lines that deduced from amino acid composition. γ-Glu-Glu residues are shown in red.

LDH aggregation at 43 °C, as monitored by light scattering at 340 nm, and its concentration-dependent suppression by PSP. It is noteworthy that the presence of even a 1/20 molar amount resulted in a considerable decrease in LDH aggregation. When added at a 1/10 molar ratio, the bacterially expressed *Artemia* PSP protein, which lacks glutamylation, inhibited aggregation to approximately only one-third of that inhibited by the same concentration of cyst-derived protein (Fig. 4B). The post-translational, multiple γ-glutamylation is, therefore, important for effective repression of heat-induced denaturation and aggregation of protein.

Discussion

Artemia PSP isolated from the RNA fraction of diapausing cysts contained a substantial amount of extra glutamate residues, which were not accounted for by the cDNA sequence (Table 1). Mass spectrometric analysis (Fig. 2) of the protein suggested the presence of heterogeneous polyglutamylation, discovered in tubulin subunits nearly 20 years ago [9] and later in nucleosome assembly proteins [15]. In *Artemia* PSP, however, a large number of glutamate residues were modified by the addition of single glutamate through the γ-amide bond. Although γ-glutamylation itself is known to be the branching point structure of polyglutamylation, and some tubulins are modified with the branching γ-glutamate only [16], the present modification in the *Artemia* protein is entirely distinct from polyglutamylation in that so many single glutamates are attached to glutamate residues along the main polypeptide chain, and there is no indication of further addition of glutamate to the branching glutamates. This modification provides a series of characteristic structural elements consisting of a pair of α- and γ-carboxy groups, which make this acidic protein even more acidic because of the lower pK_a of the α-carboxy groups. In these respects, the structural and functional consequences of the modification to the protein are likely to be quite different from that of polyglutamylation.

Polyglutamylation is catalyzed by a group of enzymes homologous to tubulin tyrosine ligase, which adds a tyrosine to the C-terminus of tubulins [17]. The polyglutamylation reaction consists of two steps: initiation, which adds glutamate to the γ-carboxy group of protein glutamate residues through isopeptide bonds, followed

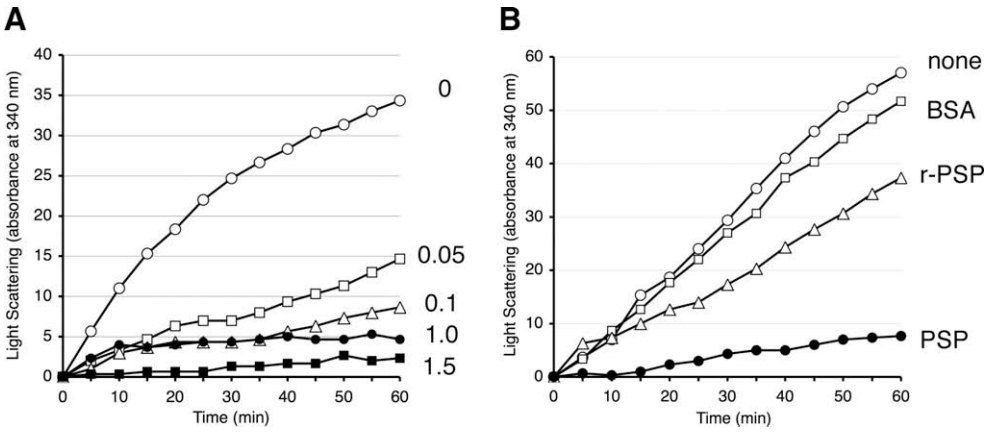

Fig. 4. Prevention of heat-induced aggregation of lactate dehydrogenase (LDH) by cyst-derived and recombinant *Artemia* PSP. (A) Effect of increasing amounts of *Artemia* cyst PSP on the thermal aggregation of rabbit muscle LDH. PSP was added at the indicated molar ratios to LDH and maintained at 43 °C. The formation of aggregation was monitored by turbidity at 340 nm ($\times 10^3$). 0, no addition. (B) Comparison of anti-aggregation activity of cyst PSP and recombinant PSP (r-PSP) at a molar ratio of 0.1 to LDH. The same concentration of bovine serum albumin (BSA) was used to assess non-specific protection. None, no addition. Data points represent the average of three separate measurements.

by elongation, which further adds glutamates to extend the polyglutamate chain through normal α-peptide bonds [18]. In the glutamylation of *Artemia* PSP, an initiation-specific enzyme may be working, but no further elongation seems to occur. In tubulins, the degree of side chain glutamate polymerization is regarded as one of the variable parameters of the modification that can modulate tubulin function depending on the length [19,20]. As shown in Supplementary Fig. S6, the pattern of modification of *Artemia* PSP is heterogeneous and seems discontinuous, at least with respect to the major modification loci. Whether this modification pattern has functional significance is not clear, but it might remind us of a kind of post-translational modification code such as the histone code [21].

LEA proteins are a group of highly hydrophilic proteins originally identified in plant seeds and thought to be responsible for the dehydration tolerance of the seeds by protecting proteins from denaturation, but they are now known to be present in many anhydrobiotic invertebrates in addition to *Artemia* [11,22]. In the protection assays, as the name suggests, LEA proteins are usually added to the test protein in abundance, 5–10-fold over the sample on a molar basis, to effect their protecting activity. By contrast, *Artemia* PSP suppressed thermal aggregation of LDH effectively even at an amount as low as a one-tenth of the enzyme. Furthermore, LEA protein usually needs the co-existence of high concentrations (around 0.1 M) of trehalose for effective suppression of thermal aggregation [7]. Therefore, *Artemia* PSP seems to be a potent suppressant of thermal stress to proteins and might participate in the extraordinary tolerance of diapausing *Artemia* cysts. Our preparation of recombinant *Artemia* PSP was not modified by glutamylation and showed rather weak suppression of thermal aggregation of LDH, suggesting that this unique PTM may be functionally important for the protection of proteins from thermal denaturation and aggregation.

In conclusion, we have discovered in diapausing *Artemia* cysts a small acidic protein heavily modified by attachment of glutamate to glutamate residues, which represents a previously unknown type of post-translational modification, multiple γ-glutamylation. Its possible protective role against heat-induced protein aggregation is suggested.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.064.

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