CANCER GENOMICS & PROTEOMICS 5: 117-122 (2008)

Galectin-3 Expression in Human Papillary Thyroid Carcinoma

PHANNEE SAWANGAREETRAKUL[1], CHANTRAGAN SRISOMSAP[1,2], DARANEE CHOKCHAICHAMNANKIT[1] and JISNUSON SVASTI[1,2,3]

[1]*Laboratory of Biochemistry, Chulabhorn Research Institute, and*
[2]*Chulabhorn Graduate Institute, Bangkok 10210;*
[3]*Department of Biochemistry and Center for Protein Structure and Function, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

Abstract. *Previous studies have suggested that galectin-3 expression was markedly elevated in papillary thyroid carcinoma compared to other thyroid diseases. In order to better understand this protein, galectin-3 from papillary thyroid carcinoma was partially purified by affinity chromatography on lactose-agarose. Proteins eluted from the column were separated by SDS-PAGE, and galectin-3 was detected with antibodies against the N-terminus and C-terminus of galectin-3. Some protein bands from the lactose binding fraction were also selected for identification by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). Seven protein bands, with molecular weights ranging from 16 kDa to 31 kDa, were identified as galectin-3. The antibody raised against the C-terminus of galectin-3 gave a strong band for one of the bands detected by the N-terminal antibody and weak bands for the other three. One additional dark immunoreactive band with an approximate molecular weight of 20 kDa, was also detected by the C-terminal galectin-3 antibody. To determine the structural differences of each protein band, N-terminal amino acid sequencing of the seven protein bands was conducted. The three upper bands were N-terminally blocked, while the other bands had N-terminal amino acid sequences starting at positions Gly35, Gly65 (2 bands) and Ala100, respectively. Further studies are necessary to determine whether these are due to nonspecific proteolysis or post-translation modification*

Thyroid carcinoma, the most common endocrine malignancy, is classified into four principle subtypes, papillary, follicular, undifferentiated, and medullary. Papillary, follicular, and undifferentiated carcinomas are derived from the follicular cells, while medullary carcinoma arises from the parafollicular C-cells of neural crest origin (1). Galectin-3 is highly expressed in thyroid carcinoma of follicular cell origin, whereas neither normal thyroid tissues, nodular goiters nor follicular adenoma express galectin-3 (2, 3). Galectin-3 was present at higher levels in papillary carcinomas than in follicular adenomas or carcinomas (4). Fine needle aspiration cytology (FNAC) of the thyroid is a rapid, minimally invasive, and cost-effective first screening for patients with thyroid nodules (5). However FNAC is unable to distinguish follicular carcinoma from its benign counterparts, such as nodular goiter and follicular adenoma. A molecular marker is required for the differential diagnosis between benign and malignant thyroid disease.

Galectins are a family of animal lectins that have two typical properties, affinity for β-galactosides and sequence homology in the carbohydrate recognition domain. Galectin-3 shows a chimeric structure, which consists of two distinct domains, an N-terminal domain containing a repetitive sequence rich in proline and glycine, and a globular C-terminal domain encompassing the carbohydrate-binding site (6). Galectin-3 previously described as IgE-binding protein, carbohydrate binding protein 35 (CBP35), CBP30, a surface antigen of mouse macrophages (Mac-2), a lectin from murine tumor cells (L-34), L-31, L-29 and a non-integrin laminin binding protein (LBP), is an endogenous β-galactoside binding protein which is expressed broadly in normal and neoplastic tissues (7). Galectin-3 is the most widely studied galectin and much is known about its structure and genomic sequence, but its precise function is unknown. Galectin-3 exerts a diversity of functions, depending on its subcellular localization. Cell surface galectin-3 mediates cell-cell and cell-matrix interaction (8, 9), while nuclear galectin-3 is involved in pre-mRNA splicing (10). Cytoplasmic galectin-3 works as an antiapoptotic molecule (11), and the expression of cytoplasmic galectin-3 is up-regulated during neoplastic progression in human malignancies, including the thyroid, colon, liver and tongue (2, 12-14).

Correspondence to: Dr. Chantragan Srisomsap, Laboratory of Biochemistry, Chulabhorn Research Institute, Vibhavadee Rangsit Road, Bangkok 10210, Thailand. Tel: +66 2 5740622, Fax: +66 2 5742027, e-mail: chantragan@cri.or.th

Key Words: Galectin-3, thyroid diseases, proteomics, immunodetection, papillary thyroid carcinoma, LC/MS/MS.

Galectin-3 has been found to be a potential marker for various carcinomas, including those of the thyroid. Previously, galectin-3 expression in thyroid disease has been studied by one dimensional electrophoresis (1-DE) immunoblotting and showed marked elevation in thyroid papillary carcinoma, compared to follicular adenoma, follicular carcinoma or non-neoplastic diseases (4, 15). Galectin-3 expression was also elevated in malignant cancer of bone, breast, colon, esophagus, larynx, lung and ovary (15). In a previous study galectin-3 expression from thyroid papillary carcinoma with metastasis gave 2-3 bands on 1-DE immunoblotting, and showed 3 dark spots with MW/pI 32.9/8.29, 31.0/8.40 and 30.0/8.40 and 2 light spots on 2-DE immunoblotting (15). However, this protein could not be identified by Coomassie blue R-250 or Sypro ruby staining after 1-DE or 2-DE, because of its low abundance in the tissue. In this study, the structure of partially purified galectin-3 from human papillary thyroid carcinoma tissue was investigated. The galectin-3 expression in various compartments was studied in an attempt to determine whether these molecules had similar structures.

Materials and Methods

Isolation of galectin from papillary thyroid carcinoma tissues. Tumor tissues (s026h/48) were collected from the Pathology Department, Pramongkutklao Hospital, Bangkok and stored at –70˚C until analysis. Adjacent tissue was formalin-fixed, paraffin-embedded and routinely examined for diagnosis. The tissues were homogenized in 5 volumes of phosphate-buffered saline (PBS) (pH 7.5), 0.15 M NaCl, 1 mM ethylenediamine tetra-acetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1x protease inhibitor cocktail with a Polytron homogenizer (IKA Labortechnik, Staufen, Germany). The homogenates were centrifuged at 15,000 rpm for 30 min. A one-tenth volume of 4 M NaCl (final concentration, about 0.5 M) and a 1/100 volume of 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were added to the supernatants recovered, and the mixtures were further centrifuged at 15,000 rpm for 30 min. The resulting supernatants were directly applied to an α-lactose agarose column (Sigma, St. Louis, MO, USA) which was equilibrated with PBS buffer, 0.5 M NaCl, 1 mM EDTA, 0.2 mM DTT, 0.01% CHAPS and 1 mM PMSF. The column was washed extensively with the same buffer used for equilibration, and then PBS buffer, 1 mM EDTA, 0.2 mM DTT, 0.01% CHAPS and 1 mM PMSF. The proteins adsorbed to the affinity resin were eluted with PBS buffer, 1 mM EDTA, 0.2 mM DTT, 0.01% CHAPS, 1 mM PMSF containing 200 mM lactose. All the steps were performed at 4˚C (16).

Protein determination. Protein content was estimated by the method of Bradford (1976) with immunoglobulin (IgG) as standard (17).

SDS-PAGE. Aliquots of the fractions eluted from the lactose-agarose affinity column (20 Bg protein) were analysed on 12% SDS-PAGE (100x105x0.75 mm). Electrophoresis was performed in a Hoefer system (Hoefer, Inc., San Francisco, CA, USA) at 12 mA, room temperature for 2 h. The protein was stained with Coomassie brilliant blue R-250.

Two-dimensional gel electrophoresis. Each sample was resuspended in lysis solution containing 9 M urea, 2% CHAPS, 2% DTT, 2% ampholine pH 3.5-10, 1 mM PMSF, 10 μg/ml pepstatin A and 5 μg/ml bestatin and incubated for 2 h at room temperature before 2-DE was performed using the immobiline/polyacrylamide system. The samples were applied by overnight in-gel rehydration of 70 mm nonlinear pH 3-10, immobilized pH gradient (IPG) strips (GE Healthcare, Buckinghamshire, UK). The first dimension, isoelectric focusing (IEF), was performed at 6500 Vh, using a Pharmacia LKB Multiphor II system (GE Healthcare). The IPG strip was equilibrated in two steps of equilibration buffer. The first step employed 50 mM Tris-HCl, buffer pH 6.8, 6 M urea, 30% glycerol, 1% SDS and 1% DTT, while 2.5% iodoacetamide replaced the DTT in the second step. The IPG strips were then applied to the second dimension 12% SDS-polyacrylamide gels (100x105x1 mm). Electrophoresis was performed at 13 mA per gel, at room temperature for 3 h. After electrophoresis, the proteins were transferred to nitrocellulose membrane by Western blotting, followed by immunodetection as described below.

Western blotting and immunodetection. The separated proteins from 1-DE or 2-DE were transferred to nitrocellulose membrane (Hybond ECL; GE Healthcare) using the Mini Trans-Blot cell (Bio-Rad, Hercules, CA, USA) at 100 V for 30 min at 4˚C. For the N-terminal galectin-3 antibody, the membrane was blocked with 5% non-fat dried milk, for 1 h at room temperature. The membrane was probed with mouse anti-galectin-3 monoclonal antibody (Research Diagnostics Inc., MA, USA) (1 μg/ml) in 20 mM Tris-buffered saline (TBS) pH 7.6, containing 0.1% Tween 20, overnight at 4˚C, repeatedly washed, once for 15 min and three times for 5 min each in the same buffer and then incubated in 1:5000 horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Dako Cytomation, Glostrup, Denmark) for 1 h. After washing, the reaction was developed using the enhanced chemiluminescence (ECL) plus detection system, with high-performance film (Hyper-film ECL; GE Healthcare).

For the C-terminal galectin-3 antibody, the blot was incubated with 1:500 diluted rabbit anti-galectin-3 polyclonal antibody (Abcam plc, Cambridge, UK) overnight at 4˚C, repeatedly washed in TBS pH 7.6, containing 0.5% Tween 20 and then incubated in 1:1000 swine anti-rabbit IgG/horseradish peroxidase (HRP) (Dako Cytomation) for 1 h. The reaction was developed as described above.

Protein identification by mass spectrometry. The protein bands from 1-DE were excised from the gel, destained and enzymatically digested by trypsin (Promega, Madison, WI, USA). The tryptic peptides were analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) (Q-TOF, Micromass, Manchester, UK) as previously described (18)

N-terminal protein sequencing. The fraction eluted with lactose from the α-lactose agarose affinity column was separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane using the 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer system. The proteins were located by staining with Coomassie Blue R-250. The N-terminal protein sequencing was performed on an ABI Procise 494 Protein Micro-sequencer (Applied Biosystems, Foster City, CA, USA) at the Protein and Proteomics Centre, Department of Biological Sciences, National University of Singapore.

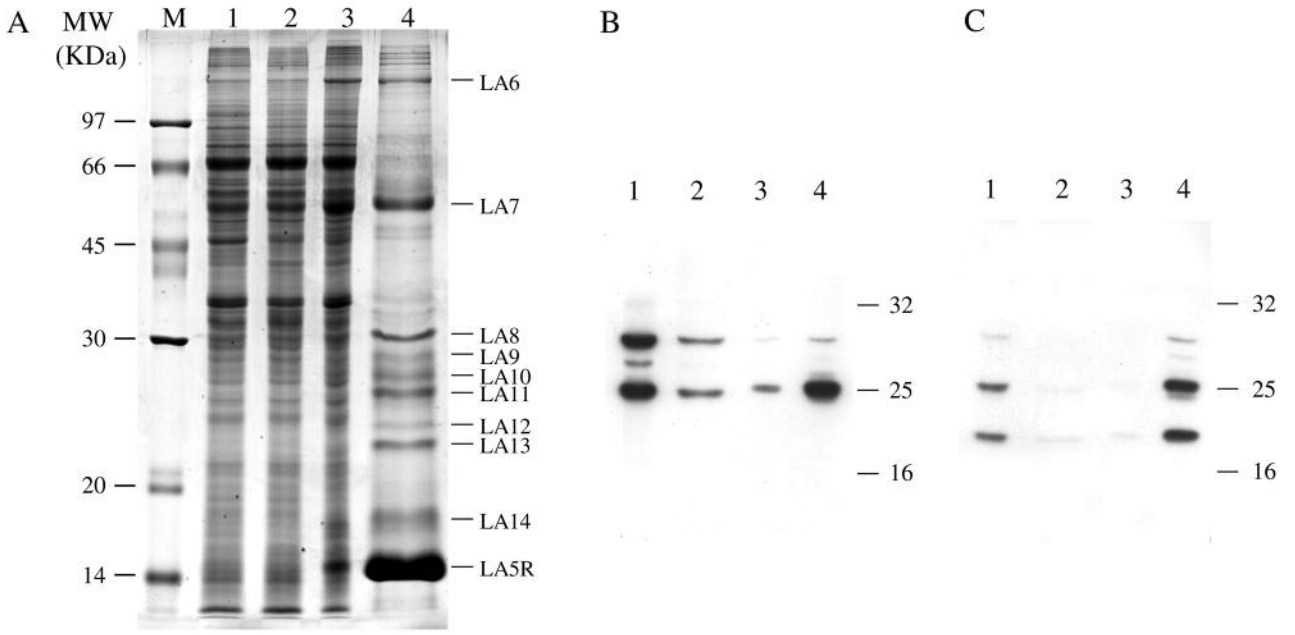

Figure 1. *1-DE pattern of protein fractions collected from alpha-lactose agarose column obtained from papillary thyroid carcinoma tissue. The proteins were separated on 12% SDS-PAGE and stained with Coomassie Blue R-250 (A). Immunoblots of the same fraction as in Figure A, labeled with the antibody to N-terminal and C-terminal of galectin-3 are shown in figure B and C, respectively. Lane M: molecular weight marker; lane 1: tissue extraction supernatant from papillary thyroid carcinoma; lane 2: flow through fraction; lane 3: wash fraction; lane 4: elute fraction with lactose.*

Results

The 1-DE SDS-PAGE analysis revealed many bands as shown in Figure 1A. Some of the major bands were excised from the gel and analyzed by mass spectrometry. The analysis revealed that seven bands named LA 8 to LA 14 corresponded to the amino acid sequence of human galectin-3, while the major band of low MW (LA 5R) was galectin-1 (Table I). To further confirm that the proteins eluted from the α-lactose agarose affinity column were indeed galectin-3 and investigate from what part of the protein they were derived, antibodies to the N-terminal and C-terminal parts of galectin-3 were used. The immunoblotting with antibody to the N-terminal of galectin-3 (Figure 1B) showed about 4 bands which corresponded to the upper 4 bands (LA 8 to LA 11). The antibody to the C-terminal part of galectin-3 (Figure 1C) recognized one strong band and three very weak bands that were the same as those detected by the antibody to the N-terminal of galectin-3. Additionally, one dark immunoreactive band of approximately 20 kDa was also detected (LA 12). The expression of galectin-3 by 2-DE immunodetection is shown in Figure 2. The expression of galectin-3 recognized by the N-terminal part and the C-terminal part of the galectin-3 antibody showed similar patterns. In addition, one dark spot of approximate MW/pI 20.0/8.4 was also detected by the C-terminal galectin-3 antibody.

The N-terminal sequences of bands LA 8 to LA 14 (as shown in Figure 1) were next analyzed. The N-terminal sequences of bands LA 8, LA 9 and LA 10 were blocked, while bands LA 11, 12, 13 and 14 revealed that galectin-3 was cleaved at amino acids 35, 65 (2 bands) and 100, respectively (Figure 3). The molecular masses calculated from the amino acid sequences of these bands were 22,571.8 for band LA 11, 19,927.67 Da for bands LA 12, LA13, and 16,854.34 Da for band LA14.

Discussion

SDS-PAGE of the partially purified lectins from thyroid papillary carcinoma revealed multiple bands of protein binding with the α-lactose agarose column. The major proteins were galectin-1 and galectin-3, but at least 7 bands were identified as galectin-3. These proteins were identified and confirmed by mass spectrometry. The antibody to the N-terminal part of galectin-3 recognized 4 bands, which corresponded to the protein bands LA 8-11 from SDS-PAGE and agreed with the galectin-3 bands seen in previous studies of thyroid disease (4, 15). In addition, the antibody to the C-terminal part of galectin-3 recognized one more protein band (LA12) that was not seen with the N-terminal antibody to galectin-3. The antibody to N-terminal part of galectin-3 used in this study was a monoclonal antibody that binds specifically to the epitope mapping to

Table I. *List of proteins identified by using LC/MS/MS.*

Band ID	Name	Peptide matches	pI/MW	Description
LA 6	ABLM2_HUMAN	1	7.81/67.77	Actin binding LIM protein 2
LA 7	30407984	1	9.23/16.88	Immunoglobulin heavy chain variable region
LA 8	LEG3 HUMAN	4	8.57/26.13	Galectin-3 *Homo sapiens*
	INADL_HUMAN	1	4.64/196.26	InaD like protein
LA 9	LEG3 HUMAN	6	8.87/26.17	Galectin-3 *Homo sapiens*
	KAC_HUMAN	2	5.50/11.60	Ig kappa chain C region
	LAC_HUMAN	1	7.07/11.23	Ig lambda chain C region
LA 10	LEG3 HUMAN	4	8.87/26.17	Galectin-3 *Homo sapiens*
	27531086	3	8.08/23.71	IgM kappa chain *Homo sapiens*
LA 11	LEG3 HUMAN	5	8.87/26.17	Galectin-3 *Homo sapiens*
	49522560	4	8.10/25.98	IGKC protein *Homo sapiens*
	PSB2_HUMAN	1	6.60/22.82	Proteasome subunit beta type 2
LA 12	LEG3 HUMAN	2	8.87/26.17	Galectin-3 *Homo sapiens*
	RL18_HUMAN	1	12.12/21.62	60S ribosomal protein L18
LA 13	LEG3 HUMAN	2	8.87/26.17	Galectin-3 *Homo sapiens*
	INADL_HUMAN	1	4.64/196.26	InaD like protein INADL *Homo sapiens*
LA 14	LEG3 HUMAN	1	8.87/26.17	Galectin-3 *Homo sapiens*
	INADL_HUMAN	1	4.64/196.26	InaD like protein
LA 5R	51464357	1	4.49/104.57	PREDICTED claudin 22 *Homo sapiens*
	37589312	1	8.66/59.68	AarF domain containing kinase1 *Homo sapiens*
	LEG1_HUMAN	1	5.14/14.71	Galectin 1 Beta galactoside binding lectin L 141
	24308303	1	5.45/43.47	SH3 binding domain protein 5 like *Homo sapiens*

the first 58 amino acids of galectin-3 while the antibody to the C-terminal part of galectin-3 was polyclonal IgG specific for amino acids 151-251 of galectin-3. So the other two bands of galectin-3 (LA 13 and 14) that did not bind to the antibody may lack the N and C terminal epitopes recognized by the galectin-3 antibodies due to proteolysis at both ends or to nonspecificity of the polyclonal antibody.

N-terminal sequencing demonstrated that the proteolytic cleavage sites in galectin-3 were not identical to previous reports of sites of cleavage by matrix metalloproteinase (MMP) (19, 20) and collagenases (21, 22). From N-terminal sequencing, band LA 8 may be the full-length galectin-3, while bands LA 9 and 10 may result from cleavage sites in the C-terminal domain. Band LA 11 lacked 34 amino acid residues at the amino terminal, but could still bind to the antibody raised against the N and C-terminals of galectin-3 and showed a stronger signal than the other 3 bands with both antibodies (Figure 1B4 and 1C4). These results suggest that the N-terminal antibody antigenic recognition site on galectin-3 is in the range between amino acid positions 35 and 58 in the collagen-like domain of the molecule because this antibody binds specifically to the first 58 amino acids of galectin-3. Band LA 12 and 13 had the same N-terminal amino acid sequence at position 65, but band LA 12 was recognized by the C-terminal antibody to galectin-3 while band LA 13 could not bind to this antibody. It was not clear for both LA 13 and 14 whether they could not recognize the C-terminal antibody to galectin-3 as a result of the nonspecificity of the polyclonal antibody or of

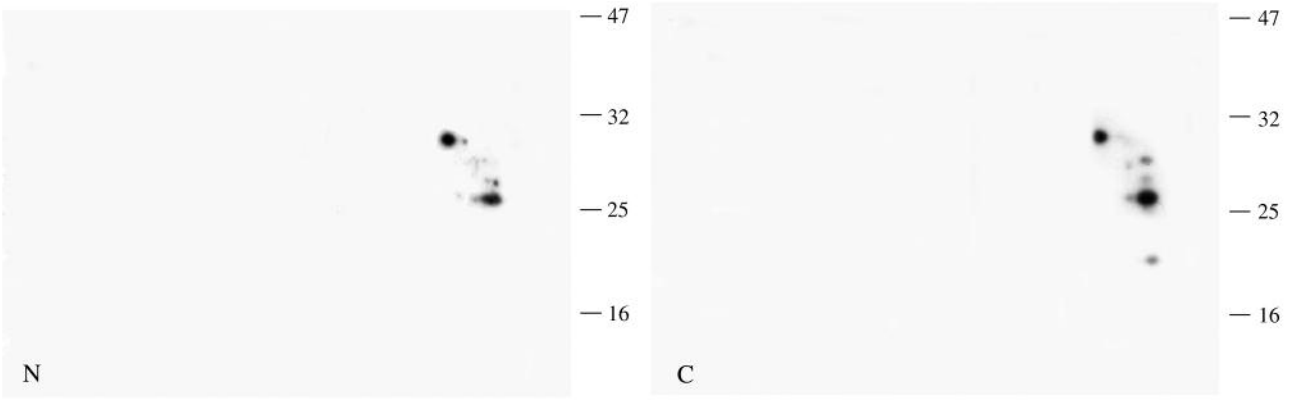

Figure 2. *Two dimensional immunodetection of eluted fraction from α-lactose agarose column using antibody to the N-terminal (N) and antibody to the C-terminal (C) of galectin-3.*

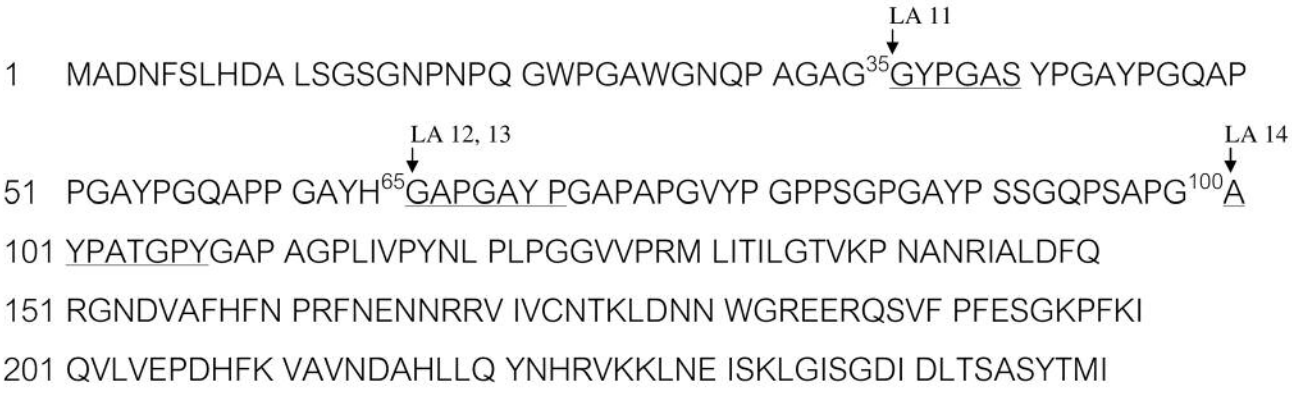

Figure 3. *Amino acid sequence of human galectin-3 (one letter code). Cleavage sites giving bands LA 11, 12, 13 and 14 are indicated in the peptide sequence of galectin-3, and the sequences obtained by N-terminal protein sequencing are underlined.*

proteolytic cleavage of the recognition site at the carbohydrate recognition domain (CRD) of galectin-3. Inspection of Figure 3 shows that each of the three novel cleavage sites at the N-terminus of galectin-3 seen in this study fell in similar sequences. Cleavage occurred in front of a small amino acid (Gly or Ala) which was preceeded by a proline five residues earlier and followed by proline two residues later ($PX_1X_2\ X_3\ X_4$*G/A X_5P, where X_1 and X_2 are small residues, G, A or S). This may indicate that a novel protease is active in papillary thyroid carcinoma, since these lower bands were previously shown to be present only in thyroid carcinoma and not in other disorders, such as goiter, hyperplasia and follicular carcinoma (15). This result suggests that these fragments of galectin-3 or the protease that generates them could be used as markers for papillary thyroid carcinoma. It is still unclear how galectin-3 is cleaved and with what type of protease. Therefore, further investigations are necessary to elucidate the enzymes responsible for the cleavage of galectin-3.

Conclusion

Galectin-1 and galectin-3 were found in the eluted fraction purified from the α-lactose agarose affinity column which is useful for enrichment of low abundance proteins such as galectin-3 in thyroid tissues, which cannot be identified from 1-DE or 2-DE Coomassie blue stained gel. Cleavage sites in front of a small amino acid (Gly or Ala) preceeded by a proline five residues earlier and followed by proline two residues later ($PX_1X_2\ X_3\ X_4$*G/A X_5P, where X_1 and X_2 were small residues, G, A or S) were suggested. The novel protease that is responsible for this action, appeared to be active only in papillary thyroid carcinoma, especially at the metastasis level (15).

Acknowledgements

We thank James R. Ketudat Cairns for helpful discussion and Phaibul Punyarit for support in collection of thyroid tissue samples. This investigation was supported by the Chulabhorn Research Institute.

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Received November 1, 2007
Revised December 20, 2007
Accepted January 9, 2008