Purification of Leucine aminopeptidase and its correlation with biochemical parameters in sera of subclinical hypothyroidism and hypothyroidism

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Abstract. The aim of this study purification of Leucine aminopeptidase (LAP) from hypothyroidism patients sera and its relation to thyroid hormones, lipid peroxidation levels of subclinical hypothyroidism and hypothyroidism patients, blood samples were collected from (50) healthy subject and (100) patients, consisted of fifty with hypothyroidism and fifty with subclinical hypothyroidism. The purification is done by addition of ammonium sulfate, dialysis, anion- exchange chromatography and size-exclusion chromatography. FT3, FT4, TSH, LAP, peroxynitrite, malondialdehyde were determined. Results showed the precipitate and concentrated protein appeared five peaks in ion exchange column, LAP activity located in the first and second protein peak, also, results showed single peak for both first and second peaks after eluted in gel filtration chromatography following steps by using SDS-polyacrylamide gel electrophoresis. From current study, it is concluded hypothyroidism (LAP) have two isoenzymes, also, concluded a highly significant increase in LAP, MDA and peroxynitrite levels in the subclinical hypothyroidism and hypothyroidism patients when compared to the healthy subject, and their correlation with FT3, FT4, TSH in the patient group, when compared by the control group that indicate these parameters could be play an effect role in these diseases.

Keywords. Leucine aminopeptidase, subclinical hypothyroidism, hypothyroidism, Lipid peroxidation

1. Introduction
Aminopeptidases could be categorized according to the substrate specificity and the chemical nature of the active site [1]. The most accurately studied aminopeptidases groups is the aminopeptidases (LAPs) that content six subunit, That back to family of metalloproteases (M17) found in all organisms [2]. Aminopeptidases are responsible for the selective release of amino acid residues from N-terminal for protein and polypeptide, Aminopeptidases are cleave a single amino acid from the amino terminal and can be used to hydrolyze polypeptides from many sources effectively [3]. Leucine aminopeptidase (EC 3. 4. 114) is a metalloenzyme contain zinc ion that is found in all organisms in different part of body. For the first time LAP was obtained as crystalline form from the bovine lens. And later hog kidney was cleansed. Kidney of swine [4]. The thyroid gland an endocrine gland in the neck, the outer and inner layer of this gland formed of follicular and parafollicular (c-cells) or thyrocyte cells [5]
It regulates heart rate, heat releasing, lipid degradation, and normal growth and development of the skeleton [6]. Biochemically, subclinical hypothyroidism is known as a high serum TSH level with a serum FT4 level about reference range [7]. Hypothyroidism is the disorder that is most severe. The disorder is known as hypothyroidism if the value of TSH is strong [8].

The oxidative damage caused by hypothyroidism and hyperthyroidism can be reduced by antioxidants, thyroid hormone involve intermediate such as a free radical which requires an ROS reaction to keeping homeostasis that's kind of the organ's function [9]. Several substances (antioxidants) are capable to protect the cells of the body against free radical damages [10] Malondialdehyde (MDA)) is one of the final products of polyunsaturated fatty acids peroxidation in the cells is commonly known as a marker of oxidative stress [11].

The purpose of this study to purification "LAP" from the hypothyroidism patients sera and its relationship to certain thyroid hormones (TSH, FT3, FT4) of subclinical hypothyroidism and hypothyroidism patients with oxidative stress that may play an effect role in these conditions.

2. Materials and Methods

2.1. Collection of subclinical hypothyroidism and hypothyroidism sera
Specimens were collected from the Specialized Center for Endocrinology and Diabetes from 1 November 2017 until the end of January 2018, blood samples were collected from (50) healthy subject and (100) patients, consisted of fifty with hypothyroidism and fifty with subclinical hypothyroidism. . The subjects were matched by body mass index (BMI) (25-29 ) Kg/m2 or less and age (15-60) years.

Determination of TSH levels [12].
Determination of T4 levels and T3 levels [13, 14].

Purification of LAP

2.2. Precipitation by using ammonium sulfate concentration 35%-75% and dialysis [15]:
Ammonium sulfate in the solid state was gradually added to the serum and the sedimentation process was carried out in a beaker with continuous stirring at about (4 °C) until the solution was turbid. After that, the fractionation of the precipitate from the supernatant was placed in a centrifuge at (4000 rpm) for a period of (20 minutes)). The precipitate was eventually dissolved in less Tris-HCl (50mM, pH 7. 0) buffer, total protein and enzyme activity assay with all concentrations.

The dialysis process was administered by adding the sample that prepared from precipitation step in dialysis tube, then this tube left dangled in a beaker containing Tris- HCl (50mM, pH 7. 0). Dialysis method was performed at (4 °C) with continuous stirring using a magnetic stirrer, while the dialysis solution was considered to be replaced during the process at the end of 24 hours. After completion this process the volume total protein, activity for final solution was measured [15].

2.3. Ion Exchange Chromatography
The column was equilibrated and washed with washing buffer, the sample from previous steps was applied to anion exchanger column" DEAE-Sepharose" with deamination (16*1. 5) cm.

After determination of flow rate about 0. 5 ml / min, each fraction was collected about 3 ml and optical density at 280 nm by used UV-visible spectrophotometer was measured. We collected the fractions that consumed the most, for these fractions, the total protein and LAP activity was measured and then eluted with 100 ml of Tris-HCl (50mM, pH 7. 0) containing a linear gradient of 0. 1–1. 0 M NaCl. . 3ml aliquot was collected. Using UV-VIS spectrophotometer, the absorbance for each fraction was measured at 280 nm, enzyme activity and total protein for fractions that gave the highest absorption has been estimated.
2.4. Gel filtration Chromatography
The previous sample that obtained from ion exchange step was passed through gel filtration column by using Sepharose 6B column with deamination (20 x 1.5) cm that wished with Tris-HCl (50 mM, pH = 7.0) buffer, collected fractions with flow rate 1ml/min each fraction was about 3ml. For each fraction, the UV-VIS spectrophotometer measured the optical density at 280 nm, enzyme activity and total protein for fractions that gave the highest absorption has been estimated.

2.5. Leucine aminopeptidase assay [16]
Leucinamide peptide bond The hydrolysis of the was measured spectrophotometrically at 238 nm. One unit of enzyme activity is equal to one micromole of L-Leucinamide hydrolyzed per minute at 25°C and pH 8.5.

2.6. Estimation of protein concentration [17, 18]
A kit provided from a company (SPINREACT) depending Biuret method was used to estimate total protein concentration.

2.7. SDS- PAGE Electrophoresis [19, 20]
The purity and molar mass of purified Leucine aminopeptidase were carried out by SDS-PAGE gel electrophoresis.

3. Results and Discussion
Leucine aminopeptidase was purified and assayed from hypothyroidism and healthy control subject Sera. Hormones of the thyroid gland and lipid peroxidation, also, determinate the correlation between them. Leucine aminopeptidase activity was evaluated [15]. Aliquot 10 ml of serum with same TSH level obtained from sera of patients with hypothyroidism was precipitated with NH₄(SO₄) and dialysis proses. Proteins are usually concentrated in the early stages of enzyme removal by removing a high percentage of water and salt to reach purity to a certain degree. Proteins are often concentrated using ammonium sulfate because they readily dissolve in water where the deposition of salts occurs as a result of neutralizing the protein charge by making salt, which leads to reduced protein dissolution and sedimentation. This is called salting out [23].

The results in Figure 1 showed five peaks of protein, four of which are in rinsing fractures and one peak in fractional washings, LAP activity and protein concentration in fractures of these five protein peaks have been determined, and the LAP data present at the first protein peak in wash fractures is in fraction numbers between (3-12),

The results in figure (1) showed five protein peaks, four of which are in the elution part and one in washing part. The highest enzyme activity and protein concentration were shown in fractions (3 - 12) in washing and (19-27) fractions that eluted at 0.1 M NaCl, but those fractions that eluted with (0.2, 0.3, 0.4)M NaCl doesn't showed activity and protein concentration. Active fractions concentrated and used for next purification steps.
3.1. Gel Filtration Chromatography

3.1.1. Wash fraction
Size exclusive chromatography was used as an important step to purification, this method depends on the size of the particles separated. The active and concentrated fraction which obtained from previous step passed through Sepharose 6B column (20 x 1.5) cm was used in this step after washed and eluted with washing buffer. Figure 2 showed the presence of leucine-aminopeptidase activity in the number of fractions (5-14), which represents the peak of one active protein. Figure (2) showed the presence of leucine aminopeptidase activity in the fractions number between (5-14) which represented with single active protein peak.

3.1.2. Elution fractions
Figure (3) showed the presence of leucine aminopeptidase activity in the fractions number between (6-15) which represented with single active protein peak.
3.1.3. SDS-PAGE Electrophoresis

The purity and molar mass of the purified LAP enzyme were determined by SDS-PAGE Electrophoresis. The results showed that the first and second peak obtained from the ion exchange chromatography step possessed LAP activity may be these peaks dimer and monomer enzyme and after applied to gel filtration
column gave about (~ 130 KD) with single band for first peak (dimer) and about ((~ 54 KD) with single band for second peak

3.2. Thyroid disorder and LAP

3.2.1. Subclinical Hypothyroidism
Table (2) shows (body mass index, thyroid hormones, leucine aminopeptidase and antioxidant) levels in the patient and control groups, the results showed a non-significant increase in BMI, FT3 and FT4 in patients group but a highly significant elevation in TSH, LAP, peroxynitrite and MDA compared to the healthy subject.

| Parameters          | Groups               | p-value |
|---------------------|----------------------|---------|
|                     | Control  n=50        | Sub-Hypo n=50 |
| BMI (kg/m²)         | 28.6±0.36            | 28.52±0.4 | P ≥ 0.05 |
| FT₃ (pmol/L)        | 6.256±1.2            | 6.41±1.66 | P > 0.05 |
| FT₄ (pmol/L)        | 14.1±3.33            | 13.27±4.09 | P ≥ 0.05 |
| TSH (muIU/ml)       | 2.32±1.48            | 9.56±1.75 | P ≤ 0.001 |
| LAP IU/ml           | 251.16±27.6          | 299.2±11.8 | P ≤ 0.001 |
| Peroxynitrite (μmol/L) | 42.68±1.87       | 60.20±1.97 | P ≤ 0.001 |
| MDA (μmol/L)        | 0.66±0.04            | 0.72±0.08 | P ≤ 0.001 |
3.2.2. Hypothyroidism

Table (3) represents (body mass index, thyroid hormones, leucine aminopeptidase and antioxidant) levels in the patient and control groups, the results showed a non-significant increase in BMI in patients group compared to healthy subject but a significant lowering in FT3, FT4, and highest significant lowering in TSH, LAP, peroxynitrite and MDA in patients group compared to the healthy subject.

| Table (3): means SE of thyroid hormone, LAP, peroxynitrite and MDA in patient and control groups |
|-----------------------------------------------|
| Parameters                  | Groups        | p-value |
|-------------------------------|---------------|---------|
|                              | Control n=50  | Hypo n=50|       |
| BMI (kg/m²)                  | 28.06±0.36    | 27.6±0.9 | P ≥ 0.05 |
| FT₃ (µmol/L)                 | 6.25±1.2      | 1.26±3.91| P ≤ 0.001|
| FT₄ (µmol/L)                 | 14.14±3.32    | 4.41±2.2 | P ≤ 0.001|
| TSH (mIU/ml)                 | 2.32±1.48     | 14.05±3.6| P ≤ 0.001|
| LAP (IU/ml)                  | 251.1±27.6    | 327±16.7 | P ≤ 0.001|
| Peroxynitrite (µmol/L)       | 42.6±1.87     | 70.0±1.86| P ≤ 0.001|
| MDA (mmole/L)                | 0.66±0.04     | 3.7±0.18 | P ≤ 0.001|

3.3. Correlation of LAP with Thyroid Hormones

3.3.1. Subclinical hypothyroidism

| Table (4): Correlation of LAP levels with thyroid hormones in the patient and control group |
|---------------------------------------------------------------|
| Parameters | Control group | Patient group |
|------------|---------------|---------------|
| LAP x FT₃ | r-value | p-value (S) | r-value | p-value (S) |
| LAP x FT₄ | 0.074 | S | 0.512 | S |
| LAP x TSH | 0.129 | S | 0.146 | S |
| LAP x FT₃ | -0.278 | S | -0.210 | S |
Figure (5): Correlation between leucine aminopeptidase activity (IU/ml) and thyroid hormones in Subclinical hypothyroidism and control groups.
In this study Leucine aminopeptidase partially purified with some steps included ammonium sulfate precipitation, DEAE- sepharose anion exchanger column which give two active peaks may be those dimmer and monomer for enzyme and finally Sepharose 4B column used as gel filtration step which give single active peak.

Aminopeptidases are classified as important photolytic enzymes because it is a role in removing amino acid residues from N-terminal peptides, and some important biological processes, some of which consist of one or two zinc ions [25].

LAPs extracted from Bacterial consist of six homosubunit but extracellular LAP found as monomer [26].
Two major peaks of LAP activity were separated by a DEAE cellulose column that obtained at least two isoforms of LAP indicated in Fruit A. deliciosa, with low molar mass according to results SDS PAGE. [27].

A group of acromegaly patients used to purify of LAP which found 2- isoenzymes of the purified LAP. It was concluded from this study that there was no difference in LAP activity between patient and control groups so LAP is not a sign of disease. [28].

In conditions such as (high blood pressure or high cholesterol level in cardiovascular disease), the TSH level is above or below the reference range. The combined results of cardiovascular disease lead to the assumption of negative results for TSH rates and thus an increased risk [29].

In patients with hypothyroidism, a positive significant correlation between TSH and HOMA-IR was observed. Thyroid dysfunction results in changes in the metabolism of glucose and lipids, which is an essential risk factor for cardiovascular diseases. In order to reduce the imminent risk, dyslipidemia and insulin resistance should be controlled aggressively [30].

In patients with hypothyroidism and hyperthyroidism, the weakness of the antioxidant system has been accompanied by increased ROS. The serum Malondialdehyde value in thyroid dysfunction patients classes evaluated substantially relative to the stable healthy subject Malondialdehyde with T3 in hypothyroidism patients and the positive non-significant association with FT4 [11].

4. Conclusion

This study amid to used sera of thyroid disorder patients to purify leucine aminopeptidase (LAP), which is partial purified by using some steps such as anion exchanger column by DEAE-Sepharose and gel filtration chromatography. Therefore, the value of serum LAP activity in patients with hypothyroidism and subclinical hypothyroidism has also increased significantly; it can be used as a diagnostic marker and has found a significant evaluated in LAP, pyroxenitrite and MDA levels in patient groups compared to healthy subject, and correlation these parameters with thyroid hormone in the patients group compared with the healthy subject group, therefore, it is considered an effective parameter in these diseases.

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