Chapter 6

ACTIVITY STATUS OF THYROID HORMONE SYNTHESIZING ENZYMES

General introduction

Thyroid hormones play an important role in cell development, growth and division. They act through their nuclear receptors and affect the transcription and translation processes. Thyroxine (T₄) and 3, 5, 3’-triiodothyronine (T₃) are the main form of hormone produced by a specialized type of thyroid cells, the follicular cells, which are devoted both to iodine uptake and hormone synthesis (Dunn and Dunn, 2000). Thyroxine (T₄) is synthesised by the follicular cells from free tyrosine and on the tyrosine residues of the protein called thyroglobulin (Tg). Iodine is captured with the "iodine trap" by the hydrogen peroxide generated by the enzyme thyroid peroxidase (TPO) (Clements and Wishart, 1956) and linked to the 3’ and 5’ sites of the benzene ring of the tyrosine residues on Tg. Upon stimulation by the thyroid-stimulating hormone (TSH), the follicular cells reabsorb Tg and cleave the iodinated tyrosines from Tg in lysosomes, forming T₄ and T₃ (in T₃, one iodine atom is absent compared to T₄), and releasing them into the circulation. Thyroxine (T₄), which is the main product of the thyroid gland, is a pro-hormone that is converted into triiodothyronine (T₃) following activation by monodeiodination. Hypo/hyper functioning of thyroid gland or more specifically, thyroid hormone level, is highly related to the three key enzymes of the thyroid hormone biosynthetic pathway, viz., TPO, (Na⁺–K⁺)-ATPase and 5’-deiodinase. Changes in the activity status of these three enzymes result in tremendous alterations in thyroid morphology and functional status.

Certain natural or artificial compounds can also be goitrogenic by directly or indirectly interfering with the thyroid gland function. Prevalence of thyroid hypofunction can be caused or aggravated by thiocyanate or flavonoids generating food items. In India, vegetarianism is dominant and culturally preferred food habit. The earlier references indicate that the related foods / drinks viz. peanut seed coat (Arachis hypogaea), moringa leaves (Moringa oleifera), spinach (Spinacia oleracea) and sugarcane juice (Saccharum sp.) may have goitrogenic / anti-thyroidal activity but the report is not available, besides these plant foods are rich in cyanogenic content or flavonoids or both which are responsible for anti-
thyroidal activity. However, available data have been insufficient to achieve complete understanding of activity status of thyroid hormone synthesizing enzymes [TPO, D1 and (Na⁺–K⁺)-ATPase] in relation to naturally occurring goitrogens viz. cyanogenic glucosides, glucosinolates, thiocyanate and flavonoids in experimental animals for prolonged duration.
Chapter 6A

(\(\text{Na}^+-\text{K}^+\))-ATPase ACTIVITY UNDER THE INFLUENCE OF THE SELECTED PLANT FOODS

Introduction

The active transport of iodide into the thyroid gland is a crucial and rate-limiting step in the biosynthesis of thyroid hormones which play an important role in the metabolism, growth and maturation of a variety of organ systems (Carrasco, 1993). Although, iodide transport into the thyroid gland has been known for decades to be mediated by a specific sodium-dependent iodide transporter located at the basolateral membrane of thyroid follicular cells, the sodium iodide symporter (NIS) gene was cloned only few years ago (Dai et al., 1996; Smanik et al., 1996). It consists of a catalytic subunit (100 000 Da) and a glycosylated subunit (50–60 000 Da) (Lingrel and Kuntzweiler, 1994). The iodine required for thyroid hormone biosynthesis in follicular epithelium of thyroid gland is accumulated through the combined actions of the (\(\text{Na}^+-\text{K}^+\))-ATPase and the Na-I-cotransporter (Dai et al., 1996). NIS co-transporters two sodium ions along with one iodide ion, with the trans-membrane sodium gradient serving as the driving force for iodide uptake. This sodium gradient, providing the energy for this transfer, is generated by the ouabain-sensitive (\(\text{Na}^+-\text{K}^+\))-ATPase. During the active transport, across the basolateral membrane of thyroid follicular cells, iodide is translocated across the apical membrane by pendrin, the Pendred syndrome gene product, which is a chloride/iodide transporter (Everett et al., 1997; Kopp, 1999; Scott et al., 1999; Royaux et al., 2000). Other apical anion transporters may also be involved. At the cell/colloid interface iodide is organified in a complex reaction involving oxidation catalyzed by thyroid peroxidase (TPO) and incorporation into tyrosyl residues along the thyroglobulin (Tg) backbone. The thyroid hormones T\(_3\) and T\(_4\) are synthesized by coupling of two iodotyrosine residues and are stored in the colloid. All of these steps are stimulated by pituitary-derived thyroid stimulating hormone (TSH), which interacts with the TSH receptor at the basolateral membrane of thyroidal cells, through the cAMP pathway. A more recent study by Riedel et al., (2001) showed that TSH not only stimulates NIS transcription and biosynthesis, but also includes evidence for posttranscriptional regulation of NIS function by TSH.

NIS-mediated iodide transport is, also inhibited by the (\(\text{Na}^+-\text{K}^+\))-ATPase inhibitor ouabain as well as by the competitive inhibitors thiocyanate and perchlorate (Carrasco, 1993).
NIS is believed to span the plasma membrane 13 times, exhibiting an extracellular NH2 terminus and a cytosolic COOH terminus. Even though the degree of NIS glycosylation appears to be different among different tissues, there are no studies showing that this alters the function and stability of NIS. Although the exact mechanisms remain to be elucidated, several hydroxy-containing amino acid residues located in transmembrane segment IX (Ser-353, Thr-354, Ser-356, Thr-357) appear to play an important role in NIS activity.

In rat NIS (rNIS), which is located upstream of the rNIS minimal promoter, recapitulates the most relevant aspects of rNIS regulation (Ohno et al., 1999). This rNIS enhancer is sufficient to confer thyroid specific expression and thyrotropin (TSH) and cyclic adenosine monophosphate (cAMP) stimulated expression of the reporter gene driven by either the NIS minimal promoter or a heterologous promoter.

However, there is emerging evidence in different countries of world that goitrogens may play a secondary role in several endemic foci. Goitrogens are chemical substances that occur primarily in plant food. Plants are known to produce more than 200,000 different bioactive natural products (also denoted secondary metabolites, specialty compounds) including cyanogens and polyphenols (Bak et al., 2006; Zagrobelny et al., 2008). Both components (cyanogens and polyphenols) are well recognised as antithyroid/goitrogenic substances (Chandra et al., 2010 and Chandra et al., 2015). Different types of cyanogens are found in plant foods viz. cyanogenic glucosides, glucosinolates, and thiocyanate (SCN).

Polyphenols/flavonoids are widely distributed in plant-derived foods such as all edible plants, nuts, grain and fruit and possess a variety of biological activities including antithyroid effects in experimental animals and humans. Polyphenols can be classified into different categories including flavonols (quercetin, kaempferol, morin, myricetin), flavonones (naringenin), flavones (luteolin, apigenin) and isoflavones (genistein) (Divi and Doerge, 1996). The relationship of the flavonoids to the human endocrine system has been reviewed by Michael Baker, (1997). It is now well recognized that flavonoids can interact with some hormone-transporting proteins and inactivating enzymes, all of which can alter the tissue concentrations of hormones such as thyroid, steroids, prostaglandins, and retinoids.

After ingestion of plants food containing goitrogen viz. cyanogenic glucosides and glucosinolates are degraded by various enzymes giving rise to a range of breakdown products viz. isothiocyanates, nitriles, epithionitriles, thiocyanates, oxazolidine-2-thiones. The first hint that anions like thiocyanate or perchlorate might act competitively on iodide transport came from an early study by Astwood, (1948) that showed that SCN induced goiter in rats. The competitive nature of the inhibition was established using thyroid slices and analysis by
double reciprocal or Dixon plots (Wolff, 1960). Similar results were obtained in simple systems such as isolated thyroid cells and vesicles (O’Neill et al., 1987).

The selected plant foods contain both the cyanogenic and polyphenolic constituents at different concentrations. Thus whether these constituents interfere the (Na⁺-K⁺)-ATPase activity in which the activity of NIS depends for impact of iodine into the thyroid gland have been investigated by feeding of those plant foods to experimental animals for different duration.

Therefore, the effects of feeding in selected plant foods viz. peanut seed coat, moringa leaves, spinach and sugarcane juice in albino rats for different durations on thyroidal (Na⁺-K⁺)-ATPase activity have been evaluated in this study. The activity of (Na⁺-K⁺)-ATPase was measured and discussed in this chapter in light of intake of dietary goitrogen (viz. cyanogenic glucosides, glucosinolates, thiocyanate and polyphenols / flavonoids) through diets and their synergistic action on it after prolonged feeding.

**Materials and methods**

**Maintenance of animals**

Described in the methodology section.

**Animal treatment**

Seventy two adult rats weighing 150±10 gm were allocated control and experimental groups. In the treatment schedule, rats were equally divided into nine groups each considered of eight rats per group. One group was kept as respective controls and fed normal laboratory diet whereas experimental rats in each group received a normal laboratory diet with one-third of the diet replaced (Chandra et al., 2006) by peanut seed coat, moringa leaves, spinach and sugarcane juice obtained from a local market in Kolkata. The animals were maintained with above mentioned regimen dividing into two sets- first set treated for 30 day and the second set treated for 60 day respectively. Feed consumption, corrected for wasted feed, and body weight were measured every seven days. Animal sacrifice and all other experimental procedures were same as described earlier.

**Measurement of (Na⁺-K⁺)-ATPase activity**

Described in the methodology section.
Results

Figure 10. (Na\(^+\)-K\(^+\))-ATPase activity of selected plant foods treated animals for 30 days and 60 days respectively. Each bar denotes mean ± SD of three pooled samples. Each pool contained a mixture of three thyroid glands isolated from three individual rats. One-way analysis of variance (ANOVA) test followed by Tukey’s post hoc test was to determine differences across means of different groups. Mean values are significantly different by ANOVA at \( p < 0.05 \). \(^a\) control 30 day versus treated 30 day group; \(^b\) control 60 day versus treated 60 day group; \(^c\) treated 30 day versus treated 60 day group.

The present study depicted marked alterations in the activity of the enzyme concerned with the synthesis of thyroid hormones following selected plant foods exposure. Thyroidal (Na\(^+\)-K\(^+\))-ATPase activity of experimental group of rats showed a considerable reduction in the 30 day and 60 day treated group when fed with selected plant foods. The decrease was more in 60 day treated group rats when compared to 30 day treated group.

There was a significant \((p<0.05)\) decrease in (Na\(^+\)-K\(^+\))-ATPase activity in the peanut seed coat (PSC) fed rats in both the group treated for 30 day and 60 day as compared to control. The decrease was more in 60 day treated group rats when compared to 30 day treated group.
Similarly, rats fed with moringa leaves showed significant decrease in (Na\(^+-\)K\(^+\))-ATPase activity. Decrease in (Na\(^+-\)K\(^+\))-ATPase activity was more evident in 60 day treated group fed with moringa leaves than 30 day treated group \((p<0.05)\).

Significant decrease in (Na\(^+-\)K\(^+\))-ATPase activity was observed in fresh spinach fed groups \((p<0.05)\) when compared to control while the values were more in 60 day treated group fed with fresh spinach than 30 day treated group \((p<0.05)\). Simultaneously, the activity of (Na\(^+-\)K\(^+\))-ATPase was decreased significantly \((p<0.05)\) after sugarcane juice administration for 30 and 60 day when compared with the control groups, with the 60-day treatment causing a more decrease in the enzyme activity.

Among all the selected plant foods, moringa leaves treated group had shown the highest inhibition followed by peanut seed coat, sugarcane juice and spinach treated groups for 30-day and 60 day respectively.

**Discussion**

In India, about 30-50% of its population is found to be vegetarian (Yadav and Kumar, 2006) and about 2,000 plant species belonging to 110 families are reported to be cyanogenic (Seigler, 1976) and 5 types of flavonoids in 90 edible plants species are already identified (Yang et al., 2008). Exposure to naturally occurring goitrogens such as cyanogenic glucosides (thiocyanate precursors) present in several foods (Ermans et al., 1983) but the content of the goitrogenic substance even in the same foodstuff show extreme differences due to genetical and ecological factors (Delange et al., 1989). Thus the present investigation has been undertaken to evaluate the goitrogenic potentiality of selected plant foods (viz. peanut seed coat, moringa leaves, spinach and sugarcane juice) by evaluating the activity of (Na\(^+-\)K\(^+\))-ATPase in thyroid gland to understand whether cyanogens and polyphenol present in the plant foods have any effect on it. Thus the effect of chronic consumption of selected plant foods by replacing one third of normal diet on thyroidal (Na\(^+-\)K\(^+\))-ATPase activity was evaluated in all the experimental groups and compared with control.

Cyanogenic glucosides are widely distributed in the plant kingdom, and more than 2500 different plant species and about 120 different glucosinolates have been reported. Cyanogenic glucosides are readily converted into the active goitrogenic agent thiocyanate by glucosidases and sulphur transferase enzymes present in plant and animal tissues (Zoller, 2007). The glucosinolates are a class of organic compounds (water soluble anions) that contain sulphur, nitrogen, and a group derived from glucose. Glucosinolates undergo a rearrangement to form isothiocyanate derivatives; isothiocyanates react spontaneously with
amino groups to form thiourea that interferes with organification of iodide and formation of thyroid hormone. Earlier studies demonstrated that the activity of (Na⁺-K⁺)-ATPase may also be competitively inhibited by isothiocyanates that are bound exclusively to the -SH group of the cysteine residue (Maloof and Soodak, 1959; Divi and Doerge, 1996; Chandra and De, 2013). This action cannot be antagonised by iodide (Dunn, 2001). Another goitrogenic substances like flavonoids can affect the function of plasma membrane transport (Na⁺-K⁺)-ATPase (Lang and Racker, 1974). The effects of flavonoids on proteins and enzymes such as topoisomerases (Na⁺ -K⁺)-ATPases, heat shock proteins, cell cycle proteins and tyrosine kinases are well known (Spanka et al, 1990). Flavonoid such as quercetin acts as a competitive inhibitor of ATP binding to the enzyme (Shoshan et al., 1980; Shoshan and MacLennan, 1981). In general, inhibition takes place through binding of the flavonoids to the ATP-binding site. Therefore, it can be assumed that iodine influx will be decreased by flavonoid exposure. They also found that the efflux of iodide was increased by all flavonoids with the exception of myricetin. This may help to explain the overall decrease in intrathyroidal iodide accumulation after exposure to flavonoids. Other mechanisms, such as the interference of flavonoids with NIS mRNA half-life, may also be relevant.

Among all the selected plant foods, moringa leaves contain highest goitrogenic constituents followed by peanut seed coat, sugarcane juice and spinach and this phenomena was responsible for the decreased activity of thyroidal (Na⁺-K⁺)-ATPase. The decrease was more in 60 days treated group rats when compared to 30 days treated group. These results indicate that the severity of selected plant foods mediated effects on (Na⁺-K⁺)-ATPase activity is also duration-dependent.

This investigation reveals that peanut seed coat, moringa leaves, spinach and sugarcane juice contains different concentration of goitrogenic constituents viz. polyphenols/flavonoids, glucosinolates and cyanogenic glucosides; after ingestion of those goitrogenic constituents were degraded by various enzymes and giving rise to a range of breakdown products which are responsible for decreased (Na⁺-K⁺)-ATPase activity in thyroid. Overall results indicate that plant foods containing goitrogens decreased the activity (Na⁺-K⁺)-ATPase that resulted in low T₃ and T₄ levels and high TSH level as found in clinical hypothyroidism.
THYROID PEROXIDASE ACTIVITY UNDER THE INFLUENCE OF THE SELECTED PLANT FOODS

Introduction

The important markers to evaluate the functional status of thyroid gland are thyroid peroxidase (TPO). The influence of the cyanogenic/polyphenol containing plant foods on thyroid peroxidase activity is reviewed in this chapter and the effects of selected plant foods in animal model have been presented and discussed based on available literature.

Thyroid peroxidase (TPO) is a glyco-hemoprotein and the molecular weight is about 60 kDa (Taurog, 1970). TPO is synthesized on polysomes is inserted in the membrane of the endoplasmic reticulum and undergoes core glycosylation. TPO is then transported to the Golgi where it is subjected to terminal glycosylation and packaged into transport vesicles along with Tg (Ericson et al., 1990). These vesicles fuse with the apical plasma membrane in a process stimulated by TSH. TPO delivered at the apical pole of thyrocytes exposes its catalytic site with the attached protoporphyrin IX containing heme protein (Taurog, 1970) in the thyroid follicular lumen (Yokoyama and Taurog, 1988). TPO activity is restricted to the apical membrane, but most of the thyroid TPO is intracellular, being located in the perinuclear part of the endoplasmic reticulum (Alquier et al., 1989; Fayadat et al., 1998). Most of this intracellular protein is incompletely or improperly folded; it contains only high mannose-type carbohydrate units, while the membrane TPO has complex carbohydrate units. Glycosylation is essential for enzymatic activity (Fayadat et al., 1998). Chronic TSH stimulation increases the amount of TPO and its targeting at the apical membrane (Penel et al., 1998). TPO is released in soluble form only after digestion of the membrane system with proteolytic enzymes (Hosoya and Morrison, 1967). The thyroid hormone synthesis is a sequential process, several tyrosine residues of thyroglobulin are first iodinated by thyroid peroxidase and then some of them are coupled to thyroid hormones by TPO (Taurog, 1970; Pommier et al., 1973). Thyroid hormone production requires the presence of peroxide, H₂O₂, iodide and the acceptor protein at one anatomic locus in the cell. The usual acceptor for iodide is thyroglobulin, which is iodinated within apical secretory vesicles at the cell border just prior to liberation into the colloid, or possibly after liberation into the colloid. The
peroxide is present in numerous cellular structures, but iodination activity occurs primarily, if not only, at the apical cell border (De groot and Niepomniszcze, 1977).

Michot et al., (1980) reported that iodide greatly stimulates the rate of bityrosine formation in the presence of thyroid peroxidase, suggesting that thyroid peroxidase contains a simple class of regulatory binding sites for iodide. SCN\(^-\) mimics iodide effects; maximal stimulatory effects were seen with about 0.5 mM thiocyanate and Km for SCN\(^-\) was equal to 0.1 mM. The effects of SCN\(^-\) and those of iodide were not additive. These results suggest that SCN\(^-\) binds to the same regulatory site as iodide but with a slightly lower affinity.

Thyrotrophin modulation of iodide uptake, H\(_2\)O\(_2\) generation, thyroglobulin synthesis and peroxide enzyme level obviously are the main regulations and many of these actions are thought to involve mediation of adenyl cyclase and subsequent activation of intracellular phosphokinase (Degroot and Niepomniszcze, 1977). Many goitrogenic constituents exert a direct effect on the thyroid gland to disrupt the biosynthesis of thyroid hormones. Inhibition of the iodide trapping mechanism is caused by thiocyanate, polyphenols, perchlorate, pertechnetate similar to iodide. Blockage of organic binding of iodine and coupling of iodothyronines to form thyroxine (T\(_4\)) and triiodothyronine (T\(_3\)) is caused by sulphonamides, thiourea, methimazole, propyl thiouracil (PTU), they react with the oxidised iodine intermediate and are irreversibly inactivated themselves and also by thiocyanate, glucosinolates etc (Capen, 1992).

Peanut seed coat (Arachis hypogaea), moringa leaves (Moringa oleifera), spinach (Spinacia oleracea) and sugarcane juice (Saccharum sp.) containing cyanogenic/polyphenolic constituents viz. cyanogenic glucosides, glucosinolates, thiocyanate and flavonoids (polyphenols) - the naturally occurring goitrogenic / anti-thyroid constituents. Cyanogenic glucosides after ingestion are readily converted to thiocyanate by glucosides and sulphur transferase enzyme present in animal tissue (Dehlberg et al., 1984).

Glucosinolates undergo a rearrangement to form isothiocyanate derivatives and thiocyanate too (Schone et al., 2001). These constituents affect hormone synthesis in thyroid gland either by inhibiting iodide uptake or interfering the activity of thyroid peroxidase i.e. by inhibiting the organification of iodide (I\(^-\) leads to I\(_2\)) or iodination of tyrosine in thyroglobulin and coupling reaction (Van Etten, 1969; Taurog, 1970; Virion et al., 1985).

Despite the apparently beneficial health effects of flavonoids, several studies indicate that they can interfere in many enzymatic systems, including those involved in thyroid hormone status (Middleton et al., 2000). It has also been shown that some flavonoids can inhibit TPO activity.
The information on the anti-thyroidal activity of the common plant foods of Indian origin is scanty. Therefore, in the present investigation attempt has been made to evaluate the thyroid peroxidase activity under the influence of selected plant foods viz. peanut seed coat, drumstick, spinach and sugarcane juice in the development of thyroid dysfunction after prolonged feeding.

**Materials and methods**

*Maintenance of animals*
Described in the methodology section.

*Animal treatment*

Seventy two adult rats weighing 150±10 gm were allocated control and experimental groups. In the treatment schedule, rats were equally divided into nine groups each considered of eight rats per group. One group was kept as respective controls and fed normal laboratory diet whereas experimental rats in each group received a normal laboratory diet with one-third of the diet replaced (Chandra et al., 2006) by peanut seed coat, moringa leaves, spinach and sugarcane juice obtained from a local market in Kolkata. The animals were maintained with above mentioned regimen dividing into two sets- first set treated for 30 day and the second set treated for 60 day respectively. Feed consumption, corrected for wasted feed, and body weight were measured every seven days. Animal sacrifice and all other experimental procedures were same as described earlier.

*Measurement of thyroid peroxidase (TPO) activity*
Described in the methodology section.
Results

Figure 11. Thyroid peroxidase (TPO) activity of selected plant foods treated animals for 30 days and 60 days respectively. Each bar denotes mean ± SD of three pooled samples. Each pool contained a mixture of three thyroid glands isolated from three individual rats. One-way analysis of variance (ANOVA) test followed by Tukey’s post hoc test was done to determine differences across means of different groups. Mean values are significantly different by ANOVA at $p < 0.05$. a control 30 day versus treated 30 day group; b control 60 day versus treated 60 day group; c treated 30 day versus treated 60 day group.

There was a significant ($p<0.05$) decrease in thyroid peroxidase (TPO) activity in the peanut seed coat (PSC) fed rats in both the group treated for 30 day and 60 day as compared to control. The decrease was more in 60 day treated group rats when compared to 30 day treated group.

Similarly, rats fed with moringa leaves showed significant decrease ($p<0.05$) in thyroid peroxidase (TPO) activity. Decrease in thyroid peroxidase (TPO) activity was more evident in 60 day treated group fed with moringa leaves than 30 day treated group ($p<0.05$). Significant decrease in thyroid peroxidase (TPO) activity was observed in fresh spinach fed groups ($p<0.05$) when compared to control while the inhibition were more in 60 day treated group fed with fresh spinach than 30 day treated group ($p<0.05$).
Thyroid peroxidase activity was decreased significantly \((p<0.05)\) after sugarcane juice administration for 30 and 60 day when compared with the control groups, with the 60-day treatment showed a more decrease in the enzyme activity.

Thyroid peroxidase activity was decreased significantly \((p<0.05)\) after supplementation of selected plant foods (viz. peanut seed coat, moringa leaves, spinach, sugarcane juice) for 30 and 60 days respectively when compared with the control groups, the 60-day treatment showed a more pronounced inhibition in the enzyme activity as compared with 30 day treatment groups.

Among all the selected plant foods, moringa leaves treated group had shown maximum inhibition followed by peanut seed coat, sugarcane juice and spinach treated groups for 30-days and 60 days respectively.

**Discussion**

The effect of chronic consumption of peanut seed coat, moringa leaves, sugarcane juice and spinach by replacing one third of normal diet on thyroid physiology was evaluated in the present study. The dietary regimen was continued for 30 days and 60 days, after the treatment period thyroid peroxidase (TPO) activity was determined and compared with the control group maintained with laboratory standardized normal diet.

Thyroid peroxidase (TPO) activity was reduced markedly in peanut seed coat, moringa leaves, sugarcane juice and spinach fed groups. Highest inhibition was noted with moringa leaves followed by peanut seed coat, sugarcane juice and spinach. Inhibition in TPO activity was noted when these foods were fed indicating that *in vivo* TPO inhibition under the influence of these plant foods was more in 60 days treated groups as expected than that of 30 treated group.

All these foods contain various concentrations of cyanogenic glycosides, glucosinolates, thiocyanate and polyphenols as was discussed in earlier chapter. Regular consumption of foods containing cyanogenic glucosides, glucosinolates, and thiocyanate affect thyroid physiology and may lead to the development of endemic goitre, especially in iodine deficient environments (Delange *et al.*, 1982).

Raben, (1949) showed that at high concentration thiocyanate inhibits the incorporation of iodide into thyroglobulin. Dahlberg *et al.*, (1984) reported the potent anti-thyroid activity of thiocyanate. Van Middlesworth (1985) found that thiocyanate increases the formation of an essentially insoluble iodinated thyroglobulin within the thyroid in iodine depleted condition, however, only a small fraction of iodination may occur through this route.
Maloof and soodak, (1959); Langer and Michajlovskij (1972) showed that iodine and thiocyanate compete at the level of thyroid peroxidase at its binding sites. The extensive work of Virion (1980) further revealed that thiocyanate inhibits the iodide oxidation i.e. conversion of $\Gamma$ leads to $I_2$ by inhibiting TPO. Iodination of tyrosine residue of thyroglobulin is also inhibited under the influence of excess thiocyanate that reacts with thyroid peroxidase. Conversely thiocyanate stimulates the coupling reaction i.e. the formation of $T_3$ and $T_4$ from MIT and DIT. Orgiazzi and Millot, (1994) reported that thyroid peroxidase is the target of thiocyanate. It blocks the iodination of tyrosine residue and coupling of iodotyrosines into iodothyronines. Greer *et al.*, (1966) found that thiocyanate has an inhibitory effect on iodination of thyroglobulin. Thiocyanate at high concentration also inhibits the incorporation of iodide into thyroglobulin by competitions with the iodide at the thyroid peroxidase level (Ermans and Bourdoux, 1989), thiocyanate or thiocyanate like compounds primarily inhibits iodide concentrating mechanism of thyroid (Capen, 1992).

Therefore, the inhibition of TPO under the influence of these plant foods was probably due to interference in TPO activity by the indigenous thiocyanate present in those plants itself and also due to the conversion of cyanogenic glucosides and glucosinolates to thiocyanate in presence of widespread glucosidases, sulphur transferases and myrosinase present in the different tissues (Reinwein, 1961; conn, 1980).

Apart from thiocyanate, some of the natural polyphenols / flavonoids inhibit thyroid peroxidase (TPO) *in vitro* and *in vivo* and in humans (1989; Gaitan, 1996). In vivo, flavonoids that are TPO suicide substrates are likely to exert a long-lasting depression of thyroid hormone synthesis because de novo enzyme synthesis is required to restore lost activity. Divi and Doerge, (1996) suggest that the inhibitory effects of flavonoids that are alternate substrate of iodination and it would be attenuated by TPO-catalyzed iodination to inactive products. However, chronic exposure of flavonoid inhibitors, especially at high doses, could elicit prolonged blockade of thyroid hormone synthesis. Any compound that causes chronic inhibition of thyroid hormone synthesis is a potential thyroid carcinogen that acts by long-term stimulation of thyroid growth induced elevated levels of thyroid stimulating hormone (TSH) (Hill *et al.*, 1989). The polyphenols/flavonoids content in plant foods cause in *in vitro* TPO inhibition which seems to be competitive; since the enzyme $V_{\max}$ was unchanged and $K_m$ for iodide was significantly increased, it likely is able to scavenge $H_2O_2$ (Divi and Doerge, 1996). Earlier studies also suggest that the consumption of foods rich in flavonoids, especially those that inactivate TPO, has the potential to induce goiter and in this manner may be involved in the etiology of human thyroid cancer (Divi and Doerge, 1996).
Our study explores that the selected plant foods containing polyphenols at different concentration might be responsible for decreased TPO activity causing decreased thyroid hormone (T₃ and T₄) levels and increased TSH levels.

Inhibition of thyroid peroxidase activity also might be for to the presence of glucosinolates in the foodstuff, because in presence of glucosinolates the action of TPO changes (Schone et al., 2001). In vitro studies of Kohler et al., (1988) demonstrates that TPO oxidizes oxazolidinethiones and probably further degradation products of glucosinolates and thus uses elemental iodine. Iodine is thus reduced to iodide which cannot be taken up by the thyroglobulin and thus interfere with the activity of thyroid peroxidase. Glucosinolates degradation product goitrin inhibits oxidation of iodide (Kohler, 1989). The thionamide like anti-thyroid effects of goitrin have also been confirmed in in vitro studies evidenced by marked inhibition of thyroid peroxidase and iodide organification (Gaitan et al., 1983; Gaitan 1986). Another degradation product of glucosinolates, isothiocyanate also interferes with organification of iodine thereby peroxidase activity might be also due to the direct action of glucosinolates or its degradation products like isothiocyanate, goitrin etc. present in the studied plants.

Moreover, cyanogenic glucosides release cyanide after ingestion. The cyanide is detoxified to thiocyanate and the thiocyanate blocks the organic binding of iodine thereby affecting thyroid peroxidase activity (Greer et al., 1966). A study of Gaitan (1990) and Chandra et al., (2013) also suggests that cyanogenic glucosides act on thyroid mainly by their rapid inhibition in TPO activity after chronic consumption of bamboo shoot rich in cyanogenic glucosides. Soybean of Indian origin containing isoflavon also inhibited thyroid peroxidase activity severely in albino rats (Chandra et al., 2004).

Finally, the inhibition of thyroid peroxidase activity as found in the present study in peanut seed coat, moringa leaves, sugarcane juice and spinach fed animals was due to the synergic action of cyanogenic glucosides, glucosinolates, thiocyanate of cyanogenic origin and polyphenols/flavonoids present in the studied plant foods. Cyanogenic glucosides, glucosinolates degraded in the animal body yielding thiocyanate, isothiocyanate, goitrin that mainly affect the activity of TPO acting at different levels while polyphenols/flavonoids act as scavenger of H₂O₂ required for oxidation of inorganic iodide to molecular iodine, for binding of iodide to the tyrosyl residues in thyroglobulin. TPO inhibition resulted in a decrease in circulation T₃ and T₄ level and subsequent increase TSH level as observed in this study.
Chapter 6C

5'-DEIODINASE-I ACTIVITY UNDER THE INFLUENCE OF THE SELECTED PLANT FOODS

Introduction

Deiodination is the most important metabolic pathway. About 100 nmol of T₄, 9 nmol of T₃ and less than 5 nmol metabolically inactive reverse T₃ (rT₃) are produced in the thyroid gland (Bianco and Larsen, 2005). T₃ differs from T₄ in that it lacks an iodine atom at the phenol ring. With regard to the position of the iodine it is important to recognize that, due to the free rotation around the ether bridge, the 5 or 3 positions of the tyrosyl ring are equivalent, and also the 3’ or 5’ positions of the phenol ring are equivalent (Bianco et al., 2002). Thus, T₄ is converted by phenolic ring deiodination (outer ring deiodination) to the bioactive hormone T₃; bioactive, because it is the principal ligand for the nuclear thyroid hormone receptors (Surks and Oppenheimer, 1977) or by inner ring deiodination to the inactive metabolite rT₃. T₃ is inactivated by inner ring deiodination to 3, 3’-diiodothyronine (3, 3’-T₂), a metabolite that is also generated by outer ring deiodination of rT₃ (Hennemann and Visser, 1997).

Three enzymes are involved in thyroid hormone deiodination and these enzymes constitute a family of selenoproteins that have been highly conserved in terms of structure and function throughout vertebrate evolution. The iodothyronine deiodinases types I, II, and III (D₁, D₂ and D₃ respectively) regulate the activity of thyroid hormone via removal of specific iodine moieties from the precursor molecule T₄. These three enzymes constitute a group of dimeric integral membrane thioredoxin fold-containing proteins (Leonard et al., 2001; Callebaut et al., 2003; Bianco and Larsen, 2005) that can activate or inactivate thyroid hormone, depending on whether they act on the phenolic or tyrosil rings of the iodothyronines, respectively (Kuiper et al., 2005).

Type I deiodinase (D₁) is expressed mainly in liver, kidney, and thyroid. The D₁ monomer is a type I integral membrane protein oriented with a 12-amino acid NH₂-terminal extension in the endoplasmic reticulum (ER) lumen and a single transmembrane domain exiting the ER at about position 36 (Toyoda et al., 1995). Among the non-sulphated conjugates, rT₃ is by far the preferred substrate, the outer ring deiodination of which is orders of magnitude faster than the deiodination of any other iodothyronine (Hennemann and Visser,
Therefore, it is not surprising that D1 is probably the primary site for the clearance of plasma rT3. Although it catalyzes the conversion of T4 to T3 much less effectively, D1 is supposed to be the major source of circulating T3 (Visser, 1988; Kohrle et al., 1991; Larsen & Berry, 1995). The conjugated compounds T4 sulphate and T3 sulphate are preferred substrates for inner ring deiodination (Germain and Galton, 1997). Dithiothreitol (DTT) is the in vitro cofactor and D1-catalyzed deiodination is sensitive to inhibition by PTU (propylthiouracil) (Oppenheimer et al., 1972). Thyroid hormone-induced stimulation of D1 activity is exerted at the transcriptional level (Berry et al., 1990; Berry et al., 1991), which in the human Dio1 gene can be attributed to the presence of 2 thyroid hormone response elements (TREs) in the 5' flanking region (FR) of the gene (Toyoda et al., 1995; Zhang et al., 1998).

D2 activity is expressed in pituitary, brain, and brown adipose tissue. D2 generates the active form of thyroid hormone T3 via deiodination of T4. Unlike D1, the role of D2 is in local T3 production. However, there is strong evidence that a significant part of plasma T3 may be generated by an extra-hepatic, PTU-insensitive mechanism, in particular in subjects with lowered plasma T4 levels.

D3 is the major T3 and T4 inactivating deiodinase, catalyzing their conversion to 3, 3'-T2 and to rT3, respectively. It is mainly expressed in placenta, pregnant uterus, brain, human embryonic liver. The actions of the deiodinases are integrated and thus promote the maintenance of serum T3 concentrations. Fluctuations in serum T4 and T3 concentrations lead to homeostatic, reciprocal changes in the activity of deiodinase.

Polyphenols / flavonoids are widely distributed in plant-derived foods, as fruits and vegetables, and have a variety of biological activities, including antioxidant (Middleton, 1984) and antithyroid effects (Lindsay et al., 1989; Divi and Doerge, 1996). It has been shown that some flavonoids can inhibit D1 activity (Spanka et al., 1990, Ferreira, 2002; Chandra and De, 2013) while flavonoids, such as fisetin, quercetin and kaempferol, stimulate D2 activity in RMS-13 cells (da-Silva et al., 2007). Synthetic flavonoids such as EMD21388 and natural plant derived flavonoids seem to inhibit T4 5'-deiodinase activity in vivo (Schroder-van et al., 1991) and are potent inhibitors of hepatic deiodinase activity in vitro (Koehrle, 2000). Diets rich in millet have also been associated with endemic goiter in parts of West Africa where millet is a staple food. In a study by Chandra and De, (2013), a decreased activity of thyroid peroxidase and 5'-deiodinase has been reported after exposure of green tea extracts. Furthermore, isoflavones have been reported to inhibit thyroid hormone biosynthesis.
at high concentrations in humans (Milerova et al., 2006); there exists a significant correlation between circulating isoflavone concentrations and thyroid gland function.

Peanut seed coat (*Arachis hypogaea*), moringa leaves (*Moringa oleifera*), spinach (*Spinacia oleracea*) and sugarcane juice (*Saccharum sp.*) are the plant foods selected for this study contain cyanogens and polyphenols/flavonoids which are metabolised in the animal body producing cyanogenic glucosides, glucosinolates, thiocyanate and flavonoids (polyphenols) – the naturally occurring goitrogenic/anti-thyroid constituents. Earlier studies suggested that thiocyanate induced hyperthyroidism enhanced deiodinase II activity in cerebellum and brain stem of rats compared to control. It is suggested that the augmentation of deiodinase II activity could be resulted for an adaptive mechanism operating in the organism in response to maintain optimal T3 levels in the brain (Lakhsmy and Rao, 1995). Previously, in experimental animals, it has been described that the high consumption of flavonoids diminished the enzymatic activity of 5'-D1 in vitro and as well as in vivo study (Ferreira et al., 2002).

Thus, the present investigation has been undertaken to evaluate the 5'- deiodinase I activity under the influence of selected plant foods viz. peanut seed coat, moringa leaves, spinach and sugarcane juice containing polyphenols as mentioned earlier after prolonged feeding.

**Materials and methods**

**Maintenance of animals**

Described in the methodology section.

**Animal treatment**

Seventy two adult rats weighing 150±10 gm were allocated control and experimental groups. In the treatment schedule, rats were equally divided into nine groups each considered of eight rats per group. One group was kept as respective controls and fed normal laboratory diet whereas experimental rats in each group received a normal laboratory diet with one-third of the diet replaced (Chandra et al., 2006) by peanut seed coat, moringa leaves, spinach and sugarcane juice respectively; obtained from a local market in Kolkata. The animals were maintained with above mentioned regimen dividing into two sets- first set treated for 30 day and the second set treated for 60 day respectively. Feed consumption, corrected for wasted feed, and body weight were measured every seven days. Animal sacrifice and all other experimental procedures were same as described earlier.

**Measurement of 5'-deiodinase- I activity**

Described in the methodology section.
Results

Figure 12. Thyroidal 5'-deiodinase I (D1) activity of selected plant foods treated animals for 30 days and 60 days respectively. Each bar denotes mean ± SD of three pooled samples. Each pool contained a mixture of three thyroid glands isolated from three individual rats. One-way analysis of variance (ANOVA) test followed by Tukey’s post hoc test was to determine differences across means of different groups. Mean values are significantly different by ANOVA at \( p < 0.05 \). \(^a\) control 30 day versus treated 30 day group; \(^b\) control 60 day versus treated 60 day group; \(^c\) treated 30 day versus treated 60 day group.

There was a significant \( (p<0.05) \) decrease in 5’ deiodinase-I (D1) activity in the peanut seed coat (PSC) fed rats in both the group treated for 30 day and 60 days respectively as compared to control. The decrease was more in 60 day treated group rats when compared to 30 day treated group.

Rats fed with moringa leaves showed significant decrease \( (p<0.05) \) in 5’ deiodinase-I activity. Decrease in 5’ deiodinase-I activity was more evident in 60 day treated group fed with moringa leaves than 30 days treated group \( (p<0.05) \).

Significant decrease in 5’ deiodinase-I activity was observed in fresh spinach fed groups \( (p<0.05) \) when compared to control while the values were more 60 day treated group fed with fresh spinach than 30 day treated group \( (p<0.05) \).
Deiodinase-I activity was decreased significantly \((p<0.05)\) after sugarcane juice administration for 30 and 60 days when compared with the control groups, with the 60-day treatment causing a more decrease in the enzyme activity.

Deiodinase-I activity was decreased significantly \((p<0.05)\) after supplementation of selected plant foods (viz. peanut seed coat, moringa leaves, spinach, sugarcane juice) for 30 and 60 days when compared with the control groups, with the 60-day treatment causing a more pronounced decrease in the enzyme activity.

Among all the selected plant foods, moringa leaves treated group had shown maximum inhibition of D1 activity, followed by peanut seed coat, sugarcane juice and spinach treated groups for 30 days and 60 days respectively.

**Discussion**

A considerable proportion of the vegetables in the human diet are derived from the cruciferous and other polyphenol containing plants. The vegetables included in this study are peanut seed coat, moringa leaves, spinach and sugarcane juice which contains both the polyphenols/flavonoids and cyanogens viz. cyanogenic glucosides, glucosinolates and thiocyanate in significant proportion.

The effect of prolonged feeding of peanut seed coat, moringa leaves, spinach and sugarcane juice replacing a portion of the normal diet respectively on thyroidal 5’dehiodinase-I activity was evaluated in all the experimental groups and compared with control.

Peanut seed coat, moringa leaves, spinach and sugarcane juice contains glucosinolates, cyanogenic glucosides and thiocyanate in high and moderate concentration as have been discussed in chapter 5. After ingestion the glucosinolates and cyanogenic glucosides; there were metabolised by various enzymes giving rise to a range of breakdown products viz. isothiocyanates, nitriles, epithionitriles, thiocyanate, oxazolidine-2-thiones etc. There were few evidences that reveal that thiocyanate might be potential for dysfunction of thyroidal 5’dehiodinase-II activity (Lakhsmy and Rao, 1999). The earlier references indicate that the related foods may have goitrogenic / anti-thyroidal activity acting at different levels other than deiodinase but the report is not available adequately, besides these plant foods are rich with flavonoids might be responsible for alteration of thyroidal 5’dehiodinase-I activity which was not assayed in earlier investigation.

The most potent natural plant-derived compounds that can affect thyroid function, thyroid hormone secretion and availability to tissues are the group of polyphenols, i.e. plant
pigments. Experimental data have shown that many flavonoids could inhibit thyroidal enzyme activity, decreasing thyroid hormones levels thus increasing TSH and causing goiter. Divi and Doerge, (1996) demonstrate that catechin showed a lower IC50 for thyroid D1 inhibition than that described for TPO, suggesting that catechin might be a more potent inhibitor of D1 than TPO activity. Intrathyroidal T4 5’- deiodination is relevant for T3 production and secretion by the thyroid. Chronic consumption of flavonoids might also induce decreased T3 serum levels because antioxidant activity of polyphenols/flavonoids may affect the peripheral conversion of thyroid hormones by way of deiodination (Chaurasia and Kar 1997; Cabrera et al., 2006, Chandra et al., 2015).

In thyroid, D1 expression is stimulated by T3, TSH and TSH receptor antibodies, where the effects of the latter are mediated by cAMP (Toyoda et al., 1990a; Toyoda et al., 1990b). However, several candidate gene association studies have reported on significant associations of single nucleotide polymorphisms (SNPs) in D1 with reciprocal changes in serum T3 versus T4 and rT3 levels (Peeters et al., 2006; Panicker et al., 2008; Medici et al., 2011).

Obviously, any change in the activity of (D1) is reflected by the changes in the concentration of T4, T3 and TSH levels in circulation. Our study suggests that the regular consumption of plant foods (viz. peanut seed coat, moringa leaves, sugarcane juice and spinach) containing goitrogens that significantly reduced the activity of D1 in the thyroid gland, resulting decreased formation of T4 from T3 levels.

As discussed earlier, the flavonoids / polyphenols content of moringa found highest followed by peanut seed coat, sugarcane juice and spinach and the D1 activity was lowest with moringa followed by peanut seed coat, sugarcane juice and spinach. Therefore, the D1 activity of the thyroid in the treated animals depends on the concentration of polyphenols / flavonoids in the plant foods. This study shows that the natural flavonoids of selected plant foods have potential for the development a state of hypothyroidism depending on the duration of exposure.