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Peptidase Activity in Shoot Tips of the Tea Plant (*Camellia sinensis* L.)

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The shoot tips of tea plants (*Camellia sinensis* L.) comprising the buds, the first two leaves and the included stems are collectively called the 'flush' in the tea industry. The flush of the tea plant is important commercially because it is the portion of the plant used in the manufacture of tea.

The first step in tea manufacture after the plucking of the flush is a process called 'withering'. In orthodox withering the flush is spread in a thin layer on tats where it is stored until the moisture content has been suitably decreased. In Ceylon, under average conditions, the moisture content of the flush is decreased from 78 to 55 % in about 18 hr. The loss of moisture, called 'physical withering', is of importance in preparing the flush for subsequent manufacturing processes (cf. Eden, 1958; Keegel, 1958; Harler, 1963), but chemical changes also occur within the tissues of the flush during this storage period and these apparently affect the quality of the final product (Trinick & Choudhury, 1963). This latter aspect of withering, called 'chemical withering', is only poorly understood at the present time and it is the subject of current investigation.

An increase in the soluble nitrogen content of tea

shoot tips during withering was reported by Evans (1929), and the observation that there was a loss of protein during the withering period was also reported (Roberts, 1939). However, it remained for Roberts & Wood (1951), using paper chromatography, to show definitely that free amino acids were formed.

Bhatia (1962*a, b*, 1963), using quantitative methods, has studied the increase of individual amino acids during withering. He has applied his techniques in comparing orthodox and faster new withering processes for their effect on chemical withering (Bhatia, 1962*a, b*, 1963).

The object of our investigation was to establish whether the changes in the concentration of free amino acids in tea shoot tips during withering was enzymic or not, and, if these changes were enzymic, to characterize the enzyme system involved. The results of our investigation are reported below.

MATERIALS AND METHODS

Source of tea shoot tips. The tea shoot tips (flush) used in the present investigation were collected from tea plants grown in a field near the Laboratory (4500 ft. elevation).

Flush was plucked and brought to the Laboratory within 1 hr. Unless otherwise stated all experiments were carried out with flush from clone TRI.2024.

Determination of total free amino nitrogen. Weighed quantities of shoot tips (usually 10 g. fresh wt.) were macerated with 80 ml. of 50% (v/v) ethanol in a blender running at full speed for 2 min. The macerated material was then refluxed for 15 min. This treatment destroyed all peptidase activity and prevented any detectable changes in the amino nitrogen concentration after sampling.

The macerated material was suitably diluted, and clarified by the addition of lead acetate. Samples of clarified extract were centrifuged at 1500g for 2 min. and 1 ml. portions of the supernatant were used for the determination of amino nitrogen according to the method of Yemm & Cocking (1955). L-Alanine (purchased from British Drug Houses Ltd., Poole, Dorset) was used as the standard.

Preparation of acetone-dried powders. Acetone-dried powders of tea shoot tips were prepared according to a modification of the procedure of Lamb & Sreerangachar (1940). A weighed quantity of tea shoot tips (usually 10 g.) was macerated in a blender running at full speed with 100 ml. of cold (-15°) 80% (v/v) acetone. After a 2 min. maceration period, the macerated material was filtered and the residue washed with 50 ml. of cold (-15°) 80% (v/v) acetone. The washed residue was returned to the blender and the process was repeated. The residue was washed and partially dried with 25 ml. of acetone. The acetone-treated powder was finally dried overnight *in vacuo* at room temperature before assay.

Assay for peptidase activity. Peptidase-activity assays were carried out with the following reaction mixture: 1.0 ml. of sodium citrate buffer, pH 5.0 (0.2M), 2.0 ml. of saturated casein solution (0.45 mg. of nitrogen/ml.) and 20.0 mg. of acetone-dried powder (approx. 1.0 mg. of nitrogen). Reaction mixtures were incubated for 1 hr. at 52° . The reaction was stopped by adding 1.0 ml. of trichloroacetic acid (20%, w/v) to the reaction mixture.

Preliminary experiments showed that under the above assay conditions the concentration of substrate was sufficient to sustain maximal activity and that the rate of reaction was proportional to the enzyme concentration to over 30 mg. of acetone-dried powder/assay mixture.

Reaction mixtures with the trichloroacetic acid solution added at the beginning of the reaction period served as blanks. No protein hydrolysis occurred under these conditions.

After the enzymic reaction was stopped, the reaction mixtures were made up to 25 ml. with distilled water and centrifuged at 1500g for 5 min. Portions (usually 1.0 ml.) of the clear supernatant were taken for the determination of free amino nitrogen by the method of Yemm & Cocking (1955).

Units of activity. One unit of peptidase activity is defined as that amount of enzyme required to hydrolyse 1 μ equiv. of peptide bonds/min. (Report of the Commission on Enzymes of I.U.B., 1961).

RESULTS

Variation of the free amino nitrogen content of tea shoot tips on withering. Typical results of measurements of the free amino nitrogen content of tea

shoot tips on withering are shown in Fig. 1. In this experiment flush was exposed to the prevailing atmospheric conditions by spreading it thinly on a table top in a well-ventilated room. This simulates commercial withering conditions.

Fig. 1 shows that there is a general increase in free amino nitrogen in the shoot tips during the normal withering period (18 hr.), but the increase is not a steady one. These results suggest that the rate of proteolysis usually exceeds the rate of respiration of amino acids under these conditions but that the relative rates of these two processes are reversed at times. This situation would be expected to occur when the experimental material is stored in an uncontrolled environment undergoing normal fluctuations.

The enzymic nature of this process was indicated by comparing normal flush with flush that had been steamed for 3 min. at the beginning of the withering period. In one experiment the increase in free amino nitrogen during an 18 hr. withering period was 0.5 and 2.9 mg. of free amino nitrogen/g. dry wt. for steamed and untreated shoot tips respectively, representing a decrease of over 80% in the increase of free amino nitrogen.

Detection of peptidase activity in cell-free preparations of tea shoot tips. Attempts to detect peptidase activity in cell-free extracts of tea shoot tips prepared by grinding them in cold (0°) 0.2M-sodium citrate buffer, pH 5.0, resulted in preparations of uniformly low activity. The activity that was found (about 2% of that found in acetone-dried powders prepared by the procedure described in the Materials and Methods section) was found in the precipitate after low-speed centrifugation (1500g for 15 min. at 0°).

Fig. 1. Variation of total amino acid content of tea shoot tips on withering. The total amino acid content (O) after different periods of withering was measured as described in the Materials and Methods section. The percentage moisture content (●) at the time of sampling represents the degree of physical withering.

Examination of acetone-dried powders used in this Laboratory for the study of polyphenol oxidase in tea shoot tips showed that they contained considerable peptidase activity, and they were used throughout the remainder of the present investigation as a cell-free source of this enzyme system.

Effect of time of incubation. When the standard assay was carried out for different times, it was found that the rate of protein hydrolysis was constant and maximal up to 1.5 hr., after which the rate appeared to become constant again at a diminished level (Fig. 2). To ensure that maximal rates of activity were measured a 1 hr. incubation period was adopted for the standard assay.

Effect of pH and buffer composition. The standard assay was modified by substituting a series of 0.2M-sodium citrate buffers at various pH values for the buffer at pH 5.0 normally used. These buffers were prepared by adding graded volumes of N-sodium hydroxide to 0.2M-citric acid solutions before making them up to volume. Exact pH values were determined after the solutions were made up to volume. A sharp pH optimum for peptidase activity was found near pH 5.0 (Fig. 3).

When the citrate buffer of the standard assay was replaced by either 0.1M-sodium phthalate buffer, pH 5.0, or McIlvaine's buffer (4.85 parts of 0.1M-citric acid to 5.15 parts of 0.2M-disodium hydrogen phosphate, pH 5.0) there was no noticeable effect on the rate of enzyme activity.

Effect of temperature. By varying the temperature of incubation used in the standard assay it was found that for a 1 hr. incubation period 52° was the optimum temperature for peptidase activity (Fig. 4).

Fig. 2. Effect of time of incubation on the level of peptidase activity. The reaction mixture was incubated for different times under standard conditions as described in the Materials and Methods section.

Fig. 3. Effect of pH on peptidase activity. Enzyme activity was determined with the standard assay system described in the Materials and Methods section except that the pH of the 0.2M-sodium citrate buffer was varied as shown.

Fig. 4. Effect of temperature on peptidase activity. Enzyme activity was determined with the standard assay described in the Materials and Methods section except that the temperature of incubation was varied as shown.

Table 1. *Distribution of peptidase activity in the parts of the tea shoot tip*

Peptidase activity was assayed as described in the Materials and Methods section.

Part of shoot tip	Specific activity (units/g. dry wt.)	Total activity (% of activity in whole shoot tip)
Bud	7.34	17
First leaf	5.95	24
Second leaf	6.14	46
Stem	4.59	13
Whole shoot tip	5.99	100

Table 2. *Peptidase activity in shoot tips of six clones of tea*

Peptidase activity was assayed as described in the Materials and Methods section. The results are given as means $\pm$ S.D. of three determinations made on samples collected on three different dates; samples were collected simultaneously from each clone.

Clone	Activity (units/g. dry wt.)
TRI. 2025	7.26 $\pm$ 1.66
TRI. 2024	6.62 $\pm$ 1.89
KEN. 16/3	5.70 $\pm$ 0
DT. 1	5.23 $\pm$ 1.68
CV. 5. B. 1	5.14 $\pm$ 1.08
PA. 22	4.52 $\pm$ 0.66

Peptidase activity in different parts of the tea shoot tip. To ascertain the distribution of the peptidase activity within the tea shoot tips, they were divided into their various parts, namely the buds, first leaves, second leaves and stems, and acetone-dried powders were made of each part. The results of the assays of these acetone-dried powders are given in Table 1. They showed that the buds contain the highest specific activity and the stems the lowest, but the most mature portion of the shoot tip tested, namely the second leaves, contained by far the greatest percentage of the total activity present in the material tested.

Peptidase activity in different clones. Samples of flush from six clones that were growing in a single field were collected on three different dates; samples were collected simultaneously from each clone. They were then compared for their peptidase activity by the standard method. The results of this study are shown in Table 2. There was a marked difference in the level of peptidase activity in the different clones examined. The maximum difference found was over 35 % (compare clones PA. 22 and TRI. 2025).

DISCUSSION

The plucked shoot tips of the tea plant can be thought of as senescent plant tissues. The enzymic

breakdown of protein is a well-known feature of senescence (Varner, 1961), and the increase of free amino acids in tea shoot tips during withering is a manifestation of a general phenomenon. The likelihood that the free amino acids formed are of importance in determining the quality of tea (Bokuchava & Popov, 1954; Nakabayashi, 1958) makes the enzyme system responsible for their formation of importance economically as well as academically. This work has been reviewed by Stahl (1962).

The method of determining amino nitrogen used in the present investigation measures all free amino groups. Therefore it is not possible, from the results reported above, to differentiate between the formation of free amino acids and the formation of polypeptides that would be soluble in 5 % (w/v) trichloroacetic acid. However, it has been shown that free amino acids are formed in intact tea shoot tips during storage after plucking (Roberts & Wood, 1951; Bhatia, 1962*a, b*, 1963), and the enzyme system studied in the present investigation is probably responsible for this phenomenon.

Attempts to obtain active preparations of the peptidase system in soluble form have not met with success as yet. The activity studied in the present investigation was always associated with material that was precipitated at low centrifugal forces (less than 1500*g* for 15 min.). Conclusions as to the soluble or particulate nature of this enzyme system are not possible, however, because of the interference caused by the high concentrations of catechins and related polyphenols present in tea shoot tips (Roberts, 1962). These substances are normally present in tea shoot tips to the extent of 20–30 % on a dry wt. basis (Sanderson, 1964), and they are known to interfere by precipitating and inhibiting enzymes (Bendall & Gregory, 1963; Hulme & Jones, 1962; Williams, 1963). The use of acetone-dried powders has the advantage of allowing the rapid separation of enzyme and cellular polyphenols; the detrimental effects of the latter substances on the enzymes is then largely prevented. However, the treatment may irreversibly insolubilize the enzymes and prevent an assessment of intercellular enzyme distribution.

The number of enzymes responsible for the tea peptidase activity investigated cannot be determined with certainty from the results obtained to date. However, the sharp pH optimum near pH 5.0 and the sharp temperature optimum at 52° that were found suggest that a single enzyme is operating. On the other hand, the change in rate of protein hydrolysis found after 1.5 hr. incubation in the standard assay is ably explained by two or more enzymes with different substrate specificities acting on a substrate containing different amounts of their respective required peptide bonds.

The difference in the level of peptidase activity in different clones of tea is apparently a genetic difference that may have considerable effect on the quality potential of a clone (Sanderson, 1964).

SUMMARY

1. The progressive increase in free amino nitrogen in tea shoot tips during withering was shown to be due to the activity of an endogenous tea peptidase system.

2. A suitable assay procedure was developed for the assay of this enzyme system in cell-free preparations of tea shoot tips.

3. The tea peptidase system showed maximal activity at 52° in 1 hr. incubations. The optimum pH was near 5.0.

4. The level of activity was markedly different in the various parts of the shoot tips and in different clones.

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Ketone-Body Synthesis in Relation to Age of Lambs

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The metabolism of butyrate by the rumen and omasum as well as by the liver of the adult sheep is known to result in the production of ketone bodies (Pennington, 1952). The pathway of synthesis is similar in rumen and omasum tissue (Hird & Symons, 1961) and in the liver of the rat (Hird & Symons, 1962). Although the physiological significance of ketone-body synthesis by any of these tissues is not yet known it is likely that such synthesis by tissues of the fore-stomachs would be related to the presence of fatty acids in the diet and their production from fermentative processes in the rumen.

The development of rumen function after the first few weeks of life is followed by increased concentrations of fatty acids in the rumen (Wardrop & Coombe, 1961) and it is therefore of interest to know whether the biochemical activity of this tissue shows changes in its metabolism of fatty acids. Accordingly, the capacity of the rumen epithelium to synthesize ketone bodies has been followed from the birth of the lamb through this dietary transition to full rumen function. Parallel studies on liver tissue from the same animals have also been made. Our results, where common types of experiments have been done, confirm the results