Effect of organic reducing agents and ferrous ion on thioglucosidase activity of *Crambe abyssinica* seed

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The addition of L-ascorbate or 2-mercaptoethanol to aged crambe seed meal tends to restore the fresh meal pattern of epi-progoitrin hydrolysis to nitriles instead of (R)-goitrin. Neither of these reducing agents has an effect on the breakdown of epi-progoitrin to goitrin by an insoluble particulate thioglucosidase from crambe meal. The addition of ferrous ion to the insoluble particles results in the conversion of epi-progoitrin to (2S)-1-cyano-2-hydroxy-3-butene instead of (R)-goitrin over a range from pH 3.9 to 6.7.

**Introduction**

Enzymatic breakdown of epi-PG in the seed meal of *Crambe abyssinica* leads to glucose, HSO₄⁻, and a variety of other products (Fig. 1) depending upon the history of the seed or upon imposed conditions of temperature or pH (1). In wetted seed meal, the typical aglucon products (other than HSO₄⁻) are three nitriles: CN-butenen (2) and two diastereomeric CN-thiobutanes (3). In certain aged or heated meals the chief aglucon product is (R)-goitrin (1). In an effort to define what factors might be responsible for such variation in products formed in crambe meal, we investigated the influence of several reagents suggested by the literature on the thioglucosidases. 2-Mercaptoethanol was studied because certain thioglucosidases have been reported to have essential SH groups (6, 7); L-ascorbate has been described as an activating agent (6-8); and ferrous ion has been implicated in various ways in the breakdown of thioglucosides (9), including nonenzymatic conversion to thionamide (10, 11).

**Materials and Methods**

**Preparation and Autolysis of Seed Meals**

Flaked seed prepared as previously described (12) was defatted by repeated extraction at room temperature with pentane-hexane, air-dried, and ground to pass a 40-mesh screen. When a thioglucoside-free meal was required, epi-PG was removed by extraction with ice-cold 80% acetone 20% 0.07 M aqueous L-ascorbate, pH 5.3, and then the meal was dried in a vacuum desiccator. Seed meals were autolysed by quickly mixing the meals with water (5-20 ml/g) and then titrating the slurry with NaOH at a constant pH (5.3) on a Radiometer titrimeter until acid production ceased. Unless otherwise stated, meals were autolysed at 25 °C.

**Preparation of Insoluble Thioglucosidase**

Twenty-five grams of seed meal were extracted at 3-10 °C, first with 10 volumes 80% acetone 20% 0.07 M ascorbate to remove part of the epi-PG, then with 10 volumes of 0.2 M NaCl 0.07 M ascorbate (pH 5.3), and finally centrifuged 10 min at 8000-15,000 x g. The centrifugate was mechanically separated.

This enzyme has been called myrosinase or glucosinolate. The naming recommended by the International Union of Biochemistry is thioglucosidase or thioglucoside glucosylhydrolase, EC 3.2.3.1 (4). For consistency with the term thioglucosidase, the substrate is called a thiogluco­side rather than the proposed name of glucosinolate (5).

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into coarse debris and fine yellow particles which were used as the enzyme. These were preserved by lyophilization.

**Assay of Germinating Seeds**

Crambe seed, freed from the loosely adherent pericarp (hull), was surface sterilized with 0.003% solution of sodium hypochlorite and allowed to germinate on moist filter paper. Germination was stopped by drying 4 h at 50 °C in a forced-draft oven. The air-dried seedlings were ground and extracted with hexane. The ground, defatted seedlings were slurried with boiling water and treated as described below for assay of aglucon products.

**Assay of Aglucon Products from epi-PG**

The autolyzed meal slurry (or aqueous mixture of insoluble thioglucosidase plus isolated epi-PG) was heated 10 min on a steam bath and then treated with 2 volumes of acetone to precipitate proteins. After centrifugation, the supernatant was freed of acetone by evaporation at 40 °C, and then extracted six times with equal volumes of ethyl ether. (R)-goitrin was measured at 244-248 µl (12). Total nitrile was measured by infrared spectroscopy at 2260 cm⁻¹ (12). Thiocyanate was measured by ultraviolet absorption at 267 µl (10, 11); when thiocyanate is measured, the aqueous supernatant must be saturated with NaCl to assure transfer to the ether phase.

A more recent gas-liquid chromatography method (13) was applied to certain of the concentrated ether extracts to measure goitrin, CN-butene, CN-thiobutane A, CN-thiobutane B, and thiocyanate simultaneously. The three cyan compounds were isolated by the Sephadex G-10 method of Daxenbichler et al. (3).

**Results and Discussion**

The products formed enzymatically from epi-PG under various conditions discussed below are summarized in Scheme 1.

**Enzymatic Products from Seed and Seed Meal**

During the germination of dehulled crambe seed, the epi-PG aglucon is converted primarily to (R)-goitrin (Fig. 2). If the ungerminated seed is crushed, another substance(s) apparently reaches the thioglucosidase and its substrate to influence the nature of the final products. In meal made from freshly harvested seed, conversion of epi-PG to nitrile was previously shown to occur at intermediate pH values, but goitrin appeared at both extremes, pH 4 and 8 (1). In meal made from aged seed, this nitrile production is partially lost (1). Meal from a 4-year-old seed autolyses to produce more goitrin than nitrile at pH 5 (Fig. 3A). The pattern of 3A is strikingly similar to the pH pattern previously found on hydrolysis of epi-PG by thioglucosidase isolated from Sinapis alba (2).

The addition of a reducing agent, such as 2-mercaptoethanol, rejuvenates aged crambe meal so that it again produces nitrile in the
TABLE I
Effect of 2-mercaptoethanol on meal autolysis at 64°C

| Aglucons, as percentage epi-PG in meal | 1 g Meal |  
| --- | --- | --- | --- | --- | --- | --- | --- |
| Goitrin | Nitrile |  
| In 10 ml water | 4.5 | 1.5 |  
| In 10 ml 0.07 M 2-mercaptoethanol | 1.6 | 3.3 |  

Changes similar to those associated with aging of meal occur more rapidly in wet meal. Upon wetting fresh meal, epi-PG initially present in the meal is converted to a mixture of CN-butene and CN-thiobutanes (3). If more epi-PG is added either to the dry meal or immediately following autolysis (at 6 min), the additional epi-PG is also converted to the cyano compounds (Table II). But if appreciable time (15 min) elapses before the addition, then the added epi-PG goes to goitrin and almost no cyano compounds are formed. This experiment shows the lability of the system converting epi-PG to the cyano compounds. In contrast to this lability in water, extraction with 80% acetone does not destroy production of cyano compounds in a meal; epi-PG added immediately to a thioglucoside-free meal is hydrolyzed to CN-butene (21%), CN-thiobutanes (18%), and goitrin (21%). The remaining 40% was not recovered in this experiment.

The reactions occurring in autolyzing crambe meal are similar to those in *Brassica napus*. Diastereomeric CN-thiobutanes are found in *B. napus* autolysates (14) just as the CN-thiobutanes are found in crambe (3). In certain ways the hydrolysis of epi-PG in crambe meal resembles the complex breakdown of glucotropaeolin in crushed *Lepidium* seeds to benzyl isothiocyanate, benzyl thiocyanate, and benzyl nitrile. For example, high temperature favors isothiocyanate formation in *Lepidium* meal (15) and goitrin in crambe meal (1) over the formation of any cyano compound. The thioglucosidase of *Sinapis alba* was reported to release the thioglucoside aglucon which then undergoes further non-enzymatic change that depends upon pH (8, 9).

TABLE II
Products from epi-PG added to crambe meal before and after autolysis

<table>
<thead>
<tr>
<th>Time of adding epi-PG</th>
<th>Products†</th>
<th>Percentage recovery from added epi-PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added to dry meal</td>
<td>Goitrin</td>
<td>CN-butene</td>
</tr>
<tr>
<td>0</td>
<td>25.5</td>
<td>21.6</td>
</tr>
<tr>
<td>Added 6 min after wetting</td>
<td>0</td>
<td>24.6</td>
</tr>
<tr>
<td>Added 15 min after wetting</td>
<td>47.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Added 30 min after wetting</td>
<td>68.5</td>
<td>6.2</td>
</tr>
</tbody>
</table>

*Enzymatic hydrolysis products from the aglucon moiety. One gram defatted meal plus 20 ml H₂O, 25°C, pH 5.3; 85 mg pure epi-PG added as shown. The reactions are completed in 2-4 min.
†Calculated as milligrams epi-PG. The values shown exclude products derived from the epi-PG initially present in the meal.
A reasonable hypothesis to account for the complex products formed in crambe meal is that the factor(s) required for production of cyano compounds is easily oxidized and unstable at either extreme of the pH range 4–8. The factor(s) may react with the enzymatically released thiogluco-side aglucon to form the cyano compounds. This hypothesis is in agreement with the reaction sequence proposed by Miller (9), but the existence of separate labile enzymes cannot be ruled out.

Preparation of Insoluble Thioglucosidase

Crambe enzyme is relatively insoluble in water, 0.1 M citrate (pH 6.0), 0.035 M 2-mercaptoethanol, or 0.5 M NaCl (pH 5.0–6.0). For example, simple extraction of the meal with 0.07 M 2-mercaptoethanol at pH 7.5 solubilizes approximately 10% as much enzyme activity as remains insoluble. The activity of a typical insoluble preparation at pH 5.3 is 0.45 μeq/min per milligram in the presence of 0.07 M ascorbate, which increases the enzyme activity. The insoluble particles produce only goitrin from the aglucon of epi-PG at pH 5.3. Neither 2-mercaptoethanol (0.06 M) nor ascorbate (0.07 M) restores nitrile production to the insoluble enzyme as they do to aged meals. The enzyme activity is completely destroyed by 5 min of heating at 75 °C.

Effect of Ferrous Ion

Miller (9) found that S. alba thioglucosidase in hydrolyzing sinigrin produces some allyl cyanide in the presence of Fe²⁺, but the pH was not specified. Austin et al. (11) extended this observation to the hydrolysis of epi-PG by S. alba enzyme at pH 5.3. They noted that CN-butene is formed in the presence of Fe²⁺. Because of the possible implication of ferrous ion in the enzymatic hydrolysis of epi-PG, the effect of Fe²⁺ was investigated in the insoluble thioglucosidase preparation from crambe.

Insoluble thioglucosidase in the presence of ferrous ion at pH 5.3 produces no goitrin from epi-PG; nitrile appears instead. This effect of ferrous ion, from either FeSO₄·(NH₄)₂SO₄ or FeSO₄, is quite specific. None of the following salts suppressed goitrin formation nor led to nitrile production: 0.001 M FeCl₃, 0.002 M CoCl₂, 0.002 M MnSO₄, 0.01 M SnCl₂, or 0.001 M NiSO₄·(NH₄)₂SO₄. Cupric salts, 0.002–0.01 M, completely inhibited the enzyme reaction. Since these metal ions have oxidation-reduction potentials which span that of Fe²⁺, a direct reducing effect of Fe²⁺ is ruled out.

Complete suppression of goitrin formation by Fe²⁺ appears over the pH range 3.9–6.7 in a system composed of 150 mg insoluble thioglucosidase preparation and 50 ml of 0.01 M FeSO₄·(NH₄)₂SO₄ in water. However, the nitrile that is produced enzymatically in this system is not the mixture found in autolyzed meal, but is only CN-butene as was shown by Sephadex G-10 separations. Formation of CN-butene in the presence of Fe²⁺ does not depend simply upon hydrogen ion concentration, and therefore differs from the pH dependence of allyl nitrile production from sinigrin in the absence of Fe²⁺ (8, 9, 16). Ferrous ion is required only in catalytic amounts to produce CN-butene enzymatically (Fig. 4). The concentration of epi-PG used was 1.25 × 10⁻² M or 10 times the highest concentration of Fe²⁺. No correction was made for the nonenzymatic conversion of epi-PG to thionamide that was observed by Austin et al. (10, 11). Whether any thionamide arises as a result of enzyme action is not known, but it must be less than 7% of the epi-PG (see Fig. 4).

The amount of Fe²⁺ required to yield predominately nitrile is of the same order of magnitude as the amount of total iron (of unknown oxidation state) found in crambe meal. Crambe meals contain from 176 to 263 p.p.m. total iron, or, at a dilution of 1 g meal plus 10 ml H₂O₂, about
3 x 10^{-4} M total iron. Of this, 50 p.p.m. or 1 x 10^{-4} M iron are picked up in the process of milling seed in iron equipment; the average iron assay of seed conventionally ground was 50 p.p.m. higher than the same seed ground in porcelain.

In spite of the presence of a sufficient quantity of iron, its involvement in whole meal autolysis could not be directly demonstrated. EDTA added to meal presumably chelates iron, but EDTA (0.04 M) added to fresh crambe meal before autolysis had no effect upon the products formed from epi-PG. All nitrile and no goitrin were formed both in the presence and absence of EDTA.

In conclusion, the enzymatic system converting the aglucon of epi-PG to cyano compounds is labile. It is destroyed by changes in pH, by aging of meal, by heat, and by aging in solution. The addition of 2-mercaptoethanol or L-ascorbate to aged crambe meal restores the fresh meal pattern of nitrile formation (see Fig. 3). Neither reducing agent has any effect on the breakdown of epi-PG to goitrin by an insoluble thioglucosidase preparation (see Scheme 1). This conversion to goitrin by insoluble enzyme is, however, suppressed by ferrous ion; CN-thiobutanes appear instead of goitrin. The effect of Fe^{2+} is specific, and is operative over nearly the same pH range as is the nitrile formation that occurs in a fresh meal. This fact is suggestive that iron initially present in crambe meal may act as a cofactor for thioglucosidase, but the formation of CN-thiobutanes remains unexplained. Further work is needed to resolve the mechanism of epi-PG breakdown in crambe meal.

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