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Multiple forms of a glucoamylase inhibitory factor from *Aspergillus niger* and its elution after adsorption onto raw wheat starch

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An inhibitory factor (IF) from *Aspergillus niger*, that inhibited the action of glucoamylase on raw starch, was adsorbed tightly onto raw starch but was almost completely desorbed by 0.02 M sodium borate. The IF was a glycoprotein and was partially purified by ion exchange chromatography into three active fractions.

Key words: Aspergillus niger, *glucoamylase inhibitory factor, raw starch adsorption*

Saha and Ueda (1984) isolated a glycoprotein from *Aspergillus niger* strain 19 that inhibited the hydrolysis of raw starch by glucoamylase. The glycoprotein had a mol wt of approx. 11,000 Da and could adsorb tightly on raw starch (Towprayoon *et al.* 1988). We studied some of the physico-chemical properties of this inhibitor and found that the protein moiety was important for its adsorption onto raw starch, since its activity was markedly decreased when treated with pronase (Towprayoon *et al.* 1988). Hayashida *et al.* (1989) also reported a similar protein moiety that separated from glucoamylase I but could adsorb on to raw starch and decreased the raw starch digestion by that glucoamylase. It seemed possible that the glycoprotein inhibitor and the glucoamylase may both bind at the same site on raw starch (Towprayoon *et al.* 1990).

In this report, we describe some further properties of the glycoprotein, including its existence in multiple forms and how the glycoprotein may be removed from raw starch after its initial adsorption.

Materials and Methods

Preparation and Assay of Crude IF

The glycoprotein inhibitory factor was prepared and assayed according to the methods of Saha and Ueda (1984). One unit of IF is defined as that amount which causes a 10% inhibition of raw starch digestion by glucoamylase preparation (2.5 unit/ml) in 18 h at 30°C and pH 3.5, using a 1% (w/v) suspension of raw wheat starch as substrate.

Glucoamylase Preparation

Glucoamylase (Glucozyme, from *Aspergillus niger*, Amano Pharmaceutical Co., Nagoya, Japan) was prepared by the method described by Saha and Ueda (1984). The activity of the glucoamylase solution was adjusted to 2.5 unit/ml for raw wheat starch hydrolysis.

Partial Purification

Crude IF solution was adjusted to pH 7 with 0.5 M sodium citrate, held at 100°C for 2.5 h and the pH adjusted to 3.5 with 0.5 M HCl to precipitate the glucoamylase, which was discarded after centrifuging at 12,000 × *g* for 10 min. The supernatant, containing IF activity, was passed through an ultrafilter with a cut off at 10,000 Da under N_2 at a pressure of 2.5 kg/cm^2. The filtrate was used as a source of IF (filtrate IF).

Raw Starch Adsorption and Elution

Raw wheat starch (10 g) was suspended in 50 ml of 0.5 M sodium citrate/HCl buffer (pH 3.5) at 4°C for 10 min, centrifuged at 10,000 × *g* for 10 min and pelleted starch mixed with 50 ml of the filtrate IF solution (see above) and placed at 4°C for 30 min with occasional shaking. The suspension was re-centrifuged as above and the supernatant was used as 'washed IF' while the pellet of 'IF adsorbed starch' was washed twice by holding in 50 ml of 0.05 M sodium citrate/HCl buffer at 4°C for 10 min and then re-centrifuging. Following Medda *et al.* (1982), IF adsorbed on raw starch was eluted by adding 50 ml of 0.02 M sodium borate (the same concentration as Medda's method) to the 'IF adsorbed starch' at 4°C and stirring gently for 30 min. After recentrifuging, the supernatant was used as the 'eluted IF'.

Figure 1 illustrates the steps to prepare filtrate IF, washed IF and eluted IF.

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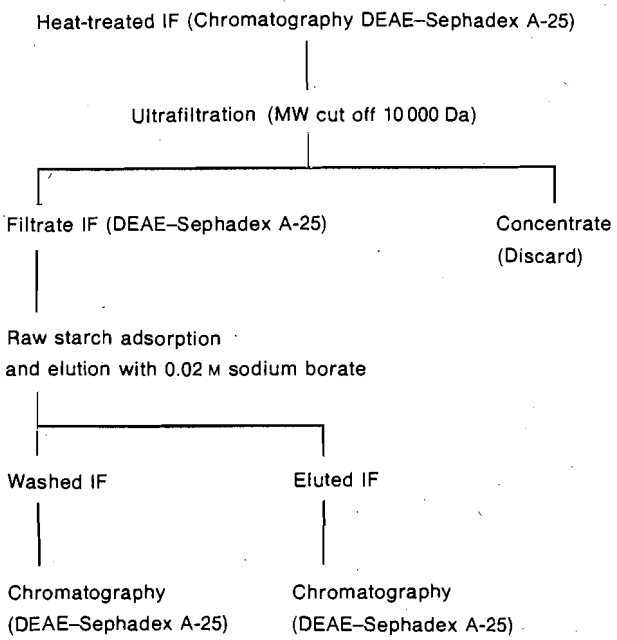

Figure 1. Protocol of sample preparation of inhibitory factor (IF).

DEAE Sephadex Column Chromatography

Heat-treated IF was applied to a DEAE Sephadex A-25 column equilibrated with 0.01 M ammonium acetate buffer (pH 5) and was eluted with a linear gradient of 0.0 to 1.0 M NaCl in the same buffer. Filtered IF, washed IF and eluted IF were applied on the same column and eluted stepwise with the same buffer containing first 0.5 M NaCl and then 0.6 M.

Protein Determination

Protein concentrations were determined by Lowry's method using bovine albumin as standard.

Results

Heat-treated IF was prepared by the method of Saha and Ueda (1984) (Table 1). Heat-treated IF, without glucoamylase activity, was separated into four components by ion exchange column chromatography (Figure 2). The heat-treated IF was also fractionated by membrane filtration (see Figure 1) and IF activity was observed in both the filtrate and the concentrate (Table 2) but with the majority being in the filtrate (filtrate IF).

Adsorption of IF onto Raw Starch and Elution with Sodium Borate

Filtrate IF was adsorbed onto raw wheat starch and was eluted with 0.02 M sodium borate following the procedure of Medda *et al.* (1982). The fractions of eluted IF and washed IF were applied to chromatography columns (see Figure 1). Table 3 summarizes the results: 75% of IF activity, (filtrate

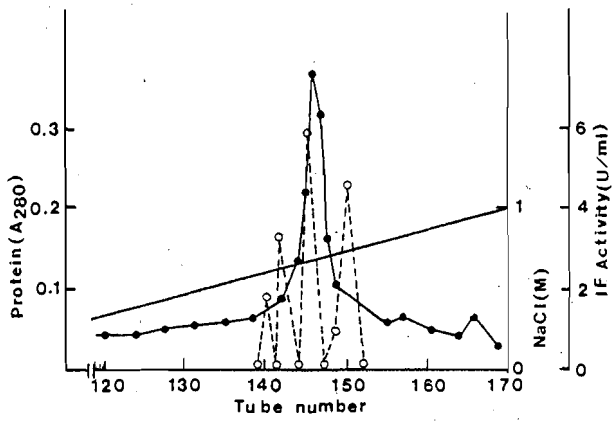

Figure 2. Chromatogram of heat-treated inhibitory factor (IF) applied on DEAE Sephadex A-25 with linear gradient of 250 ml of 0.0 to 1.0 M NaCl. Column size 2.5 × 45 cm; flow rate: 18 ml/h; fractions: 6 ml/tube. ●—Protein; ○—IF activity.

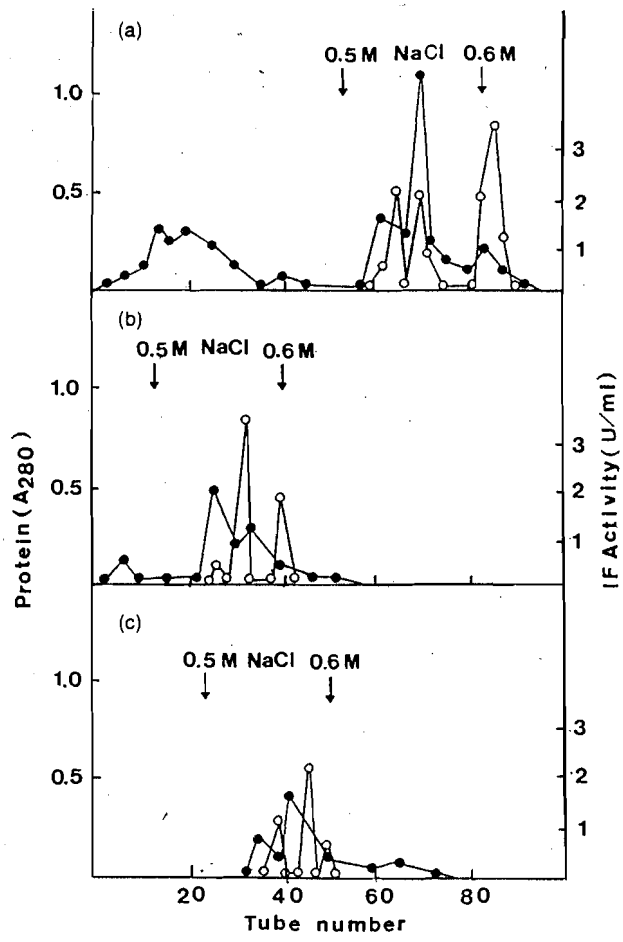

Figure 3. Elution profiles of (a) inhibitory factor (IF) filtrate, (b) washed IF and (c) eluted IF applied on DEAE Sephadex A-25 chromatography columns and eluted with 75 ml of 0.5 M and 75 ml of 0.6 M NaCl. Column size 1.5 × 30 cm; flow rate: 20 ml/h; fractions: 3 ml/tube. ●—Protein; ○—IF activity.

Table 1. Summary of heat-treated inhibitory factor (IF) from culture fluid of *A. niger* strain 19*.

	Volume (ml)	Protein (mg/ml)	Total protein (g)	Glucoamylase activity (unit/ml)	IF activity (unit/ml)	Total IF activity (unit)
Culture	2000	9.8	19.6	5.5		
$(NH_4)_2SO_4$	500	20.1	10.1	12		
EtOH	300	24.8	7.4	15		
Heat-treatment	200	4.9	1.0	0.0	2.6	520

* Towprayoon *et al.* (1988).

Table 2. Summary of ultrafiltration of crude (heat-treated) inhibitory factor (IF).

	Volume (ml)	Protein (unit/ml)	Total protein (mg)	IF activity (unit/ml)	Total IF activity (unit)	Specific activity	Purification (fold)
Crude IF (heat-treated)	100	4.9	490	2.6	260	0.5	1.0
Concentrated IF (MW > 10000)	30	14.2	425	1.6	48	0.1	0.2
Filtrated IF (MW < 10000)	70	0.6	38	2.5	175	4.6	8.6

* Based on specific activity of crude IF.
IF activity yields of concentrated IF and filtrated IF were 19 and 68% respectively, based on total IF activity in crude IF.

Table 3. Summary of raw wheat starch adsorption of filtrate inhibitory factor (IF) and elution with sodium borate.

	Volume (ml)	Protein (mg/ml)	Total protein (mg)	IF activity (unit/ml)	Total IF activity (unit)	Specific activity (unit/ml)
Applied: filtrate IF (MW < 10000)	50	0.6	27.4	2.5	125	4.5
Recovered:						
Washed IF	46	0.4	14.2	0.7	31.6‡	2.2
Eluted IF	50	0.2	9.0	1.7	85.0	9.4
Total Recovery			23.2*		116.6†	

* Based on filtrate protein, protein recovery of washed IF was 52%, eluted IF was 33% and total recovery was 85%.
† Based on filtrate IF activity, activity recovery of washed IF was 25%, eluted IF was 68% and total recovery (washed IF + eluted IF) was 93%.
‡ Based on filtrate activity, adsorption % onto raw wheat starch was 75%.

Table 4. Summary of filtrate inhibitory factor (IF), washed IF and eluted IF applied on DEAE Sephadex A-25 chromatography.

	Filtrate IF			Washed IF			Eluted IF		
	PI	PII	PIII	PI	PII	PIII	PI	PII	PIII
Total protein (mg)	3.9	9.4	2.5	7.1	5.1	1.0	0.6	2.4	0.4
Total activity (unit)	8.9	24.6	42.0	1.6	22.0	5.7	3.9	8.0	2.3
Specific IF activity (unit/ml)	2.3	2.6	17.0	0.2	3.7	5.8	6.9	3.3	5.2

PI, PII and PIII indicate IF activity peaks on chromatogram (Figure 3).

IF minus washed IF)/filtrate IF, could be adsorbed onto raw starch, of which 91% (based on adsorbed IF activity) was eluted with 0.02 M sodium borate.

Chromatography of Filtrate IF, Washed IF and Eluted IF

Filtrated IF, eluted IF and washed IF fractions (Table 3) were applied to DEAE Sephadex A-25 columns. These three fractions each gave three peaks (Figure 3) of which the first and second peaks were eluted with 0.5 M NaCl and the third peak was eluted with 0.6 M NaCl. The results are summarized in Table 4, which shows that the protein in filtrate IF was recovered completely (105%) in three peaks of washed and eluted IF. However, only 43 units of IF activity in filtrate IF were recovered out of the 75 units total activity of the three peaks of washed IF and of eluted IF. As a result, the multiforms of IF may be supported by the facts shown in Figure 3 and Table 4.

Discussion

On the basis of IF activity, 68% has an M_r below 10,000 Da. 75% of it can be adsorbed onto raw wheat starch of which 91% can be eluted with 0.02 M sodium borate. As IF can be separated into 3 fractions by DEAE Sephadex chromatography this supports the multiform nature of the IF and is different from the result of Saha and Ueda (1984), in spite of these authors using the same strain as ourselves.

Medda *et al.* (1982) obtained 89% elution of glucoamylase I adsorbed onto raw wheat starch using 0.02 M sodium borate. In this paper, 0.02 M sodium borate was also effective in eluting IF from IF adsorbed raw wheat starch. This result seems to indicate the same desorption mechanism (Medda *et al.* 1982) occurs for the present IF and glucoamylase I activity. However, the similarity between glucoamylase I and the IF is not completely revealed at present.

Acknowledgment

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