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Preliminary communication

Pancreatic alpha-amylase inhibitors in cereals*

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Cereal grains, in common with the seeds of many other plants, contain proteinaceous inhibitors of alpha-amylases[1]. The most extensively studied cereal alpha-amylase inhibitors are those from wheat, but the presence of an alpha-amylase inhibitor in rye has also been demonstrated[2,3]. Early studies on an alpha-amylase inhibitor preparation from wheat showed it to act on both salivary and pancreatic alpha-amylases, the former being the more strongly inhibited[2,3]. More recent work has provided evidence for a multiplicity of alpha-amylase inhibitors in wheat extracts, and has indicated that individual inhibitors differ in specificity for alpha-amylases from different sources. Thus, Shainkin and Birk[4] isolated two inhibitors from wheat which differed in their activities towards mammalian and insect alpha-amylases; one inhibitor acted on both types of alpha-amylase, whereas the second inhibited only insect alpha-amylase. Recently, however, it has become clear that the situation is more complicated and that a total of three or four alpha-amylase inhibitors are present in wheat[5,6]. The inhibitors are distinguished not only in terms of their relative activities towards insect and mammalian alpha-amylases, but also by apparent differences in inhibitory activity towards salivary and pancreatic alpha-amylases. Thus, four inhibitor fractions obtained by O'Donnell and McGeeney[6] had SAI:PAI** activity ratios of 30:1, 4:1, 2:1, and 1:1. All inhibitor preparations from cereals hitherto studied inhibit salivary alpha-amylase at least as strongly as pancreatic alpha-amylase, and usually much more so. For example, Strumeyer[7] and O'Donnell and McGeeney[6] have described inhibitors with at least 100-times more SAI activity than PAI activity. Since crude extracts of wheat and other cereals contain similar levels of SAI and PAI activity[8], it might be inferred that inhibitors having greater activity towards pancreatic alpha-amylase than towards salivary alpha-amylase must also exist. We now report evidence for the presence of such inhibitors in wheat and rye.

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*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

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**Abbreviations: SAI, salivary alpha-amylase inhibitor; PAI, pancreatic alpha-amylase inhibitor.

Initial efforts to demonstrate the presence of specific PAI(s) in cereal extracts were performed with hog pancreatic alpha-amylase that had been immobilized by coupling[9] to cyanogen bromide-activated Sepharose 4B. When limited amounts of immobilized alpha-amylase were added to extracts of wheat and rye that had been heat-treated at 70° for 15 min to inactivate beta-amylase, selective binding of PAI activity took place. Immobilized alpha-amylase (~10 mg bound to 2 ml of gel) was added to a solution (5 ml) of a heat-treated extract of wheat (cultivar Stewart) containing 740 units/ml of SAI activity and 208 units/ml of PAI activity (amylase inhibitor activity units are defined in Ref. 8) in 20 mM acetate buffer (pH 5.5) containing 4mM calcium chloride. After centrifugation, the supernatant solution was assayed for PAI and SAI activities. This treatment resulted in a decrease in the specific activity of PAI of 82% and a much smaller decrease (7%) in SAI specific activity. However, it was never possible to obtain completely selective binding of PAI activity. The futility of efforts to isolate a specific PAI by batchwise treatment of crude extracts with immobilized pancreatic alpha-amylase, in the manner described, was quickly recognized. Although the cereal inhibitors described previously have a preference for salivary alpha-amylase, all are able to inhibit pancreatic alpha-amylase to some degree, so that they will also tend to bind to the immobilized pancreatic enzyme. Therefore, further efforts to demonstrate, definitively, the existence of specific PAI(s) were performed by column-chromatographic procedures.

Heat-treated extracts of wheat and rye were applied to columns of immobilized pancreatic alpha-amylase and thoroughly washed to remove unbound protein. Washing the column with ethanol (66%), to reverse the interaction of inhibitors with the immobilized alpha-amylase (*cf.* Ref. 3), resulted in elution of a sharp peak of SAI activity, which also contained PAI activity. Prolonged washing with alcohol resulted in recovery of PAI activity which had an apparently much greater affinity for the immobilized alpha-amylase. This latter activity was not associated with any SAI activity. Similar results were obtained with both wheat and rye. These observations supported the preliminary evidence for the existence of a specific PAI in cereal extracts, but the procedure was not considered convenient for isolation of the PAI because of the prolonged alcohol-wash required to recover the inhibitor, and the poor yields of PAI obtained.

Having demonstrated what appeared to be a specific PAI, attempts were then made to isolate it by more convenient, conventional purification procedures. An extract of rye (cultivar Prolific), after heat treatment and centrifugation, was applied to a column of CM-cellulose equilibrated with acetate buffer (10mM, pH 4.0). SAI and PAI activities bound to the column and were recovered by elution with a gradient of sodium chloride; both activities were present in the same column fractions. The partly purified inhibitor preparation was then chromatographed on a column of Sephadex G-100; the distributions of SAI and PAI activities in the column effluent were again found to be coincident. The combined inhibitor-containing fractions, after dialysis and concentration, were then chromatographed on a column of DEAE-cellulose (DE-52, Whatman Biochemicals) equilibrated with citrate–phosphate buffer (5mM, pH 8.0). Inhibitor activity was

recovered without the application of a salt gradient, and two active fractions were apparent (Fig. 1). The first, which was slightly retarded by the ion-exchanger, inhibited only pancreatic alpha-amylase; the second, more strongly retarded, fraction contained both SAI and PAI activity.

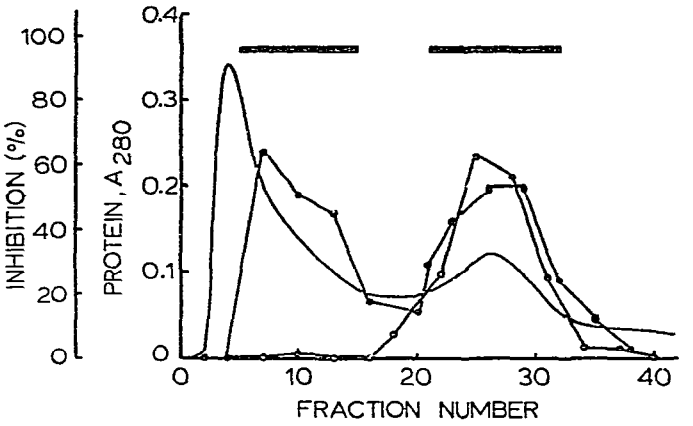

Fig. 1. Chromatography on DEAE-cellulose of a partly purified preparation of alpha-amylase inhibitor from rye; column (30 × 1.5 cm) equilibrated with 5mM citrate–phosphate buffer (pH 8.0). Protein was located by absorbance at 280 nm (——), and activities towards human salivary and hog pancreatic alpha-amylases were performed as described elsewhere[8]; –○–, inhibition of salivary alpha-amylase (5 μl of each fraction used); –●–, inhibition of pancreatic alpha-amylase (20 μl of each fraction used). The fractions under the bars were combined.

Detailed studies on the specificities and properties of the two inhibitors will require further investigation of the optimal conditions for their interaction with alpha-amylase (temperature, pH, preincubation conditions, *etc.*; *cf.* Refs. 8, 10, and 11). However, preliminary indications of the specificities of the two inhibitors are given in Table I. The second inhibitor fraction from DEAE-cellulose chromatography has specificity similar to inhibitors that have been described previously from wheat, namely high activity towards salivary alpha-amylase and *Tenebrio molitor* alpha-amylase, and considerably less activity on pancreatic alpha-amylase. The first inhibitor fraction from DEAE-cellulose differs in specificity from other known inhibitors of alpha-amylase by virtue of its much higher activity towards pancreatic alpha-amylase than salivary alpha-amylase. This inhibitor may be of value in the specific determination of pancreatic alpha-amylase in physiological fluids. Further studies on its properties are in progress.

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TABLE I

SPECIFICITIES OF TWO ALPHA-AMYLASE INHIBITORS FROM RYE

Source of alpha-amylase	*Relative inhibitor activity*[a]	
	Fraction 1	*Fraction 2*
Hog pancreas	++++	++
Human pancreas	++++	++
Human saliva	±	+++++
Tenebrio molitor	+++++	++++
Bacillus subtilis (saccharifying amylase)	++	++++
Helix pomatia	±	++++
Bacillus amyloliquefaciens	0	0
Aspergillus oryzae	0	0
Rye	0	0
Barley malt	0	0

[a]Fractions 1 and 2 refer to the first and second inhibitor fractions from DEAE-cellulose column chromatography (Fig. 1). The alpha-amylases indicated (~0.04 International Unit of each) were incubated with the same amount (5 μl) of each inhibitor fraction at 25° for 30 min, and the residual activity was determined. Key: +++++, complete inhibition; ++++, strong inhibition (greater than 70%); +++, moderate inhibition (40–70%); ++, low inhibition (10–40%); ±, slight inhibition (less than 10%); 0, no inhibition.

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