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Separation of coeliac-active peptides from bread wheat with the aid of methylpyrrolidinone chitosan

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Gliadin-derived peptides from haxaploid wheat, known to be toxic for in-vitro cultured small-intestinal mucosa from coeliac patients, are very active in agglutinating K562(S) cells. Chromatographic data obtained with the use of methylpyrrolidinone chitosan coupled to Sepharose-6B indicate that a minor (1·3%) fraction of the peptides binds very strongly to the support at nearly neutral pH values, and can be eluted at pH 2·85. This fraction is the one responsible for the agglutination of K562(S) cells, being one-hundred times more active than the digest from which it has been separated.

INTRODUCTION

In coeliac disease, genetic and environmental factors play a role in prolamin damage to the small-intestine mucosa. The mechanism by which prolamin-derived peptides produce jejunal lesions is not fully understood (Weiyers & Van de Kamer, 1960; Cornell & Townley, 1973; Strober, 1978). It was suggested that coeliac disease is a metabolic defect expressed as an increase of incomplete oligosaccharide chains, not normally associated with the surface membrane glycoproteins of the mature intestinal epithelial cells, that allows gliadin-derived peptides to act as a lectin thus initiating cell toxicity (Weiser & Douglas, 1976). It was shown by Kottgen *et al.* (1982) that the Frazer's gluten fraction III binds to brush border glycoproteins of immature crypt cells of rat intestine and much less to brush border glycoproteins from enterocytes of the mature villous zone. Moreover, a highly significant correlation between toxicity of various cereal prolamins for coeliac patients and the agglutinating activity of undifferentiated K562(S) cells has been indicated by Auricchio *et al.* (1984*a*, 1986 and 1990). The K562(S) cells are a subline isolated from the outgrowth of a clone from a patient with chronic myelogenous leukemia (Lozzio & Lozzio, 1975) that respond to treatment to hemin or butyric acid undergoing in-vitro pseudo-erythroid differentiation (Cioè *et al.*, 1981). The proteins and peptides which are toxic *in vivo* and *in vitro* to coeliac small intestine (namely, bread wheat gliadins and gliadin peptides; prolamins and prolamin peptides from rye, oats and barley; and some A-gliadins) can damage the in-vitro developing fetal rat intestine and agglutinate K562(S) cells at very low concentration. At the same time, the peptides that are non-toxic *in vivo* and *in vitro* for coeliacs (namely, bread wheat albumin, globulin and glutenins; prolamin peptides from rice and maize; and some other A-gliadin peptides) are unable to damage the in-vitro developing fetal rat intestine and to agglutinate K562(S) cells (de Ritis, 1979; Auricchio *et al.*, 1982, 1984*b*). Therefore, the toxic activity for the coeliac intestine of a large series of proteins, a mixture of

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peptides and pure A-gliadin peptides correlates very well with the cell agglutinating activity. This suggests that the unknown mechanism on which the agglutinating activity of certain gliadin peptides is based, may be involved in the pathogenesis of the coeliac intestinal lesion.

During the last few years, the K562(6) cells have been used not only to study the mechanism of mucosal damage, but also to identify toxic proteins and peptides. Mannan and oligomers of 2-acetamido-2-deoxyglucose prevent the cell agglutinating activity and the in-vitro damaging effect of the active peptides on the developing fetal rat intestine and on the flat coeliac mucosa (Auricchio *et al.*, 1987, 1990). Mannan and *N*-acetylglucosamine oligomers bind all the peptides of a gliadin digest that agglutinate K562(S) cells and that damage in-vitro fetal rat intestine (Auricchio *et al.*, 1993).

The scope of the present work was to separate active peptides from hexaploid wheat with the aid of a suitable chitin derivative, by affinity chromatography. Highly hydrophilic and biocompatible methylpyrrolidinone chitosan, which contains a substantial proportion of *N*-acetylglucosamine units, was used in this work (Muzzarelli, 1993).

EXPERIMENTAL

Methylpyrrolidinone chitosan

Chitin from *Crangon crangon* (100 g, particle size 185–355 μm) was contacted with hydrogen peroxide (1600 ml, 15 vol.) for 24 h, in order to partially depolymerize it. Chitin was from the same lot previously characterized by Focher *et al.* (1992). The bleached chitin powder was then washed with demineralized water (10 liters) and dried at 35°C; yield was 90 g. Chitosan, obtained by deacetylation at 75°C, had degree of deacetylation 0·766, as determined by first-derivative UV spectrophotometry; overall yield was 53 g. 5-Methylpyrrolidinone chitosan was then synthesized according to a proprietary procedure (Muzzarelli, 1992; Muzzarelli *et al.*, 1993). The viscosity of the 1·0% chitosan solution was 13 mPa s, instead of 48 mPa s for the control (unbleached). The identity of chitin and its derivatives was monitored by infrared spectrometry.

Methylpyrrolidinone chitosan–Sepharose-6B

The coupling of 5-methylpyrrolidinone chitosan to the Sepharose-6B gel was performed as recommended by Pharmacia (Uppsala, Sweden) with a slight modification. The reaction mixture was coupled at 37°C for 48 h, then the gel was charged with 0·1 M ethanolamine at pH 8 and kept at 4°C overnight. The coupling efficiency was determined with the colorimetric method for sugars described by Lever (1972) and found to be 57%.

Wheat gliadin and digest

Pure hexaploid bread wheat (*Triticum aestivum*, variety S. Pastore) was kindly supplied by Istituto Sperimentale Cereagricoltura, Sant'Angelo Lodigiano, Milan, Italy. Wheat gliadin fraction was extracted as described by Auricchio *et al.* (1982) and was submitted to peptic and triptic sequential digestion according to de Ritis *et al.* (1979), to obtain the peptic-triptic digest (PT-digest) which was submitted to heating for 30 min at 100°C.

Affinity chromatography

Wheat gliadin PT-digest (*c.* 50 mg) was percolated at a flow rate of 24 ml/h through a methylpyrrolidinone chitosan–Sepharose-6B column (5 cm × 35 cm) equilibrated with 0·02 M ammonium acetate buffer, pH 7·2. Fractions A and B were collected and the column was washed with the above buffer until no absorbance at 278 nm was detected in the effluent. Fraction C was eluted from the column with 0·1 N acetic acid (introduced at eluant/bed volume ratio 1·6), after the pH value dropped to 2·87. Fraction C was immediately neutralized by addition of 0·5 M ammonium hydroxide (*c.* 450 μl/tube). The fractions coded A, B and C were separately freeze-dried and stored at −20°C until required for analysis.

Cell culture and agglutination test

The K562 subclone (S), kindly donated by Dr Cioè, was cultured as previously described (Auricchio *et al.*, 1984*a*). For the agglutination test, cells were washed and resuspended with Dulbecco phosphate buffer saline at a final concentration of 10^4 cell/ml. The test (with or without sugar) was carried out as described by Auricchio *et al.* (1984*a*).

RESULTS AND DISCUSSION

The peptic–triptic digest obtained from bread wheat gliadin under experimental conditions simulating in-vivo protein digestion, was very active in agglutinating undifferentiated K562(S) cells, in agreement with previous observations made by Auricchio *et al.* (1990). The PT-digest was resolved on a methylpyrrolidinone chitosan–Sepharose-6B column as described in Fig. 1. The total recovery of protein present in the separated fractions was 95%, as determined according to the Lowry method modified by Oyama and Eagle (1956). The results showed that fraction A, eluted in the void column volume, represented 1·0–1·5% of the total amount, while fraction B, eluted with the same buffer was about 92%; fraction C, eluted with 0·1 M acetic acid was about 1·3% of the total loaded proteins.

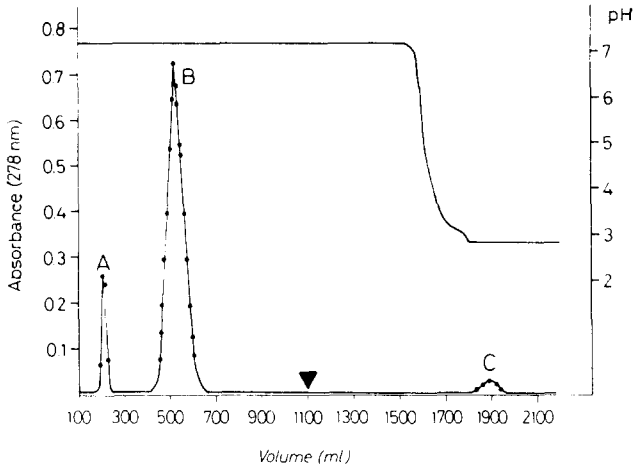
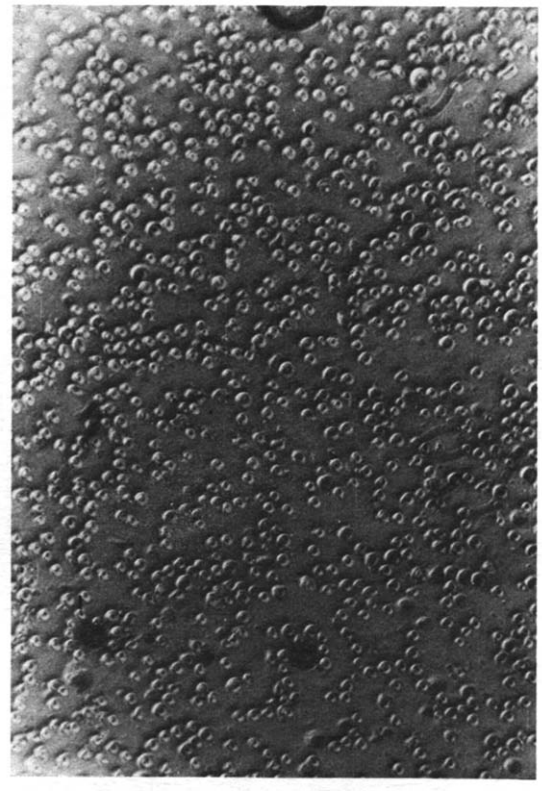
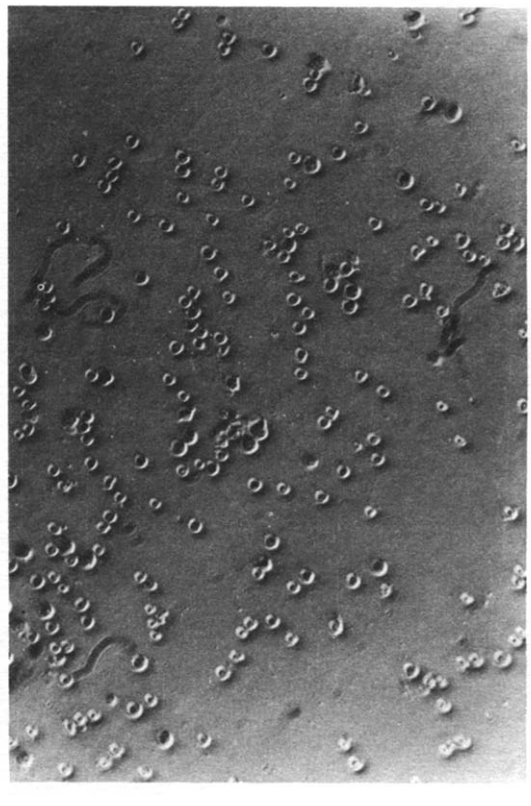


Fig. 1. Fractionation of peptic–triptic digest from bread wheat by affinity chromatography with methylpyrrolidinone chitosan–Sepharose-6B. Fractions A and B were eluted with 0·02 M ammonium acetate buffer, pH 7·2; fraction C was eluted with 0·1 M acetic acid, pH 2·8: arrow indicates change of eluent.

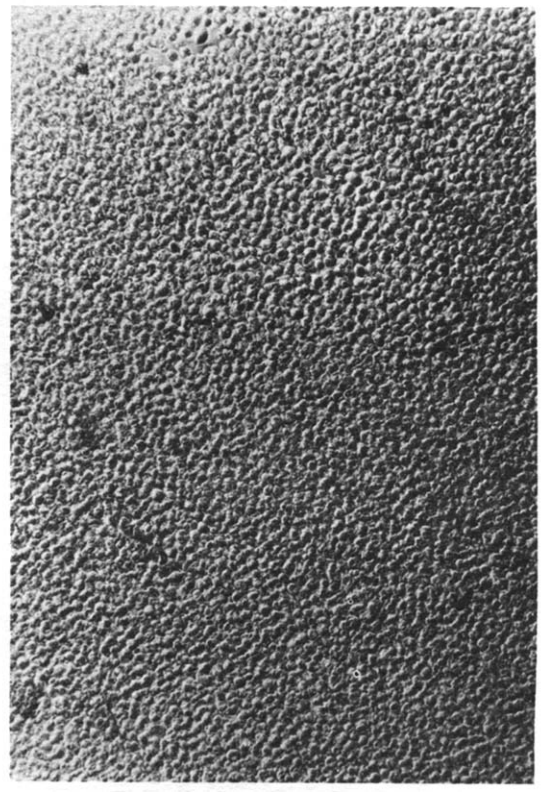
In agreement with earlier findings, no cell clustering was observed in the control undifferentiated K562(S) cells. Fractions A and B, tested at concentrations higher than 14 mg per ml of culture medium were not active (Fig. 2(a) and (b); on the contrary, about 100% of the



(b)



(a)



(c)

Fig. 2. Agglutination of undifferentiated K562(S) cells by fraction peptides from PT-digest of bread-wheat gliadin submitted to affinity chromatography (× 310). (a) Control preparation of undifferentiated K562(S) cells. (b) Same cells supplemented with 14 mg of fraction B per ml of cell suspension. (c) Same cells supplemented with 1·6 μg of fraction C per ml of cell suspension.

agglutinating activity present in the sample was recovered in fraction C. The minimal concentration required to agglutinate 100% of the cells in suspension was 1·6 μg per ml of culture medium. This fraction was about one-hundred times more active in agglutinating K562(S) cells than the PT-digest. Cells agglutinated by fraction C showed a peculiar appearance, with a tendency towards the formation of a continuous cell layer, and, when pipetted several times, had a distinct high resistance to shearing and whirling forces (Fig. 2(c)).

It is therefore concluded that methylpyrrolidinone chitosan is a suitable candidate for further in-vivo studies aimed at offsetting the action of the protein fraction responsible for coeliac disease. Recent data indicate that low molecular weight methylpyrrolidinone chitosan can be produced by enzymatic hydrolysis under conditions acceptable to the food industry (Muzzarelli *et al.*, 1994).

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