Journal of Chromatography, 434 (1988) 429–438
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4474

ISOLATION AND ANALYSIS OF PEPTIDIC FRAGMENTS OF α-GLIADIN USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

PETR KOCNA*, PŘEMYSL FRIČ and MARIE KOČOVÁ-HOLÁKOVÁ

Laboratory of Gastroenterology, Charles University, Faculty of General Medicine, Karlovo náměstí 32, CS-121-11 Prague-2 (Czechoslovakia)

JAN SLABÝ

Department of Internal Medicine, Charles University, Faculty of General Medicine, Karlovo náměstí 32, CS-121-11 Prague-2 (Czechoslovakia)

EVŽEN KASAFÍREK

Research Institute for Pharmacy and Biochemistry, Kouřimská 17, CS-130-60 Prague-3 (Czechoslovakia)

and

W.Th.J.M. HEKKENS

Laboratory of Physiology, University of the Leiden, Wassenaarseweg 62, 2300 RC Leiden (The Netherlands)

SUMMARY

Peptidic fragments of α-gliadin were obtained by peptic-tryptic-pancreatic (PTP) digestion of the α-gliadin fraction isolated by ion-exchange chromatography on a sulphopropyl-Sephadex C-50 column. The proteolytic digest was fractionated by ultrafiltration into three subfractions, $PTPa_1$–$PTPa_3$. The subfraction $PTPa_2$ was then analysed and individual peaks were separated using reversed-phase high-performance liquid chromatography (RP-HPLC) using a gradient of acetonitrile in 0.1% trifluoroacetic acid and a Separon SGX-C_{18} sorbent. A 100-mg amount of the $PTPa_2$ subfraction was separated in a single analysis by preparative RP-HPLC and twenty peaks were obtained for further characterization. The molecular mass in range 300–3000 was established for individual peptidic fragments by gel-permeation chromatography on a TSK-G2000 SW column.

INTRODUCTION

Proteins from gluten are involved in the etiopathogenesis of coeliac disease [1–3]. Different fractions isolated from gliadin were found to be toxic to patients

0378-4347/88/$03.50 © 1988 Elsevier Science Publishers B.V.

with coeliac disease. The greatest toxicity was attributed to α-gliadin [4–6], although all fractions, including ω-gliadins, are toxic [7].

Proteolytic digestions with pepsin–trypsin [8,9], followed by pancreatin [10] or intestinal peptidases [11] of gluten, gliadin or fractions prepared from gliadin, have been reported and described as PT, PTP or PTPIP digests. These fractions were tested biochemically, immunologically and in vivo by challenge tests in coeliac patients.

Further separations of peptides have been obtained on Bio-Gel P-10 [12] and by ion-exchange chromatography [10]. Currently, high-performance liquid chromatographic (HPLC) methods are used for both analytical and preparative separations. Reversed-phase HPLC (RP-HPLC) in an acetonitrile gradient was recommended by Bietz and co-workers [13–15] for the identification of gliadin and was used also for the preparation of pure toxic peptides from PT digests of β-gliadin [16] or from Frazer's fraction III [17].

This study involved the use of RP-HPLC for the analysis of peptides generated by PTP digestion of the α-gliadin and for the purification of toxic peptides in amounts of 100 mg of PTP fragments in a single analysis.

EXPERIMENTAL

Preparation of α-gliadin

The α-gliadin fraction was purified from a crude gliadin (Sigma, St. Louis, MO, U.S.A.) or the A-gliadin preparation [5]. A 5-g amount of the gliadin was separated according to Charbonnier and Mossé [18] by ion-exchange chromatography on SP-Sephadex C-50 in a glass column (100 cm×6 cm I.D.) with a flow-rate of 22 ml/h using a Varioperpex-II pump (LKB, Bromma, Sweden). The elution was monitored at 278 nm with a Uvicord-II detector (LKB) and the main peaks were analysed by starch gel electrophoresis in aluminium lactate buffer (pH 3.1) [19]. The fractions containing α-gliadin were dialysed against 15 l of 20 m*M* acetic acid in the Visking dialysis tube (Serva, Heidelberg, F.R.G.) and then concentrated by vacuum evaporation and lyophilized.

Proteolytic treatment

The peptic–tryptic–pancreatic digest (PTP fragments) was obtained by modification of the method according to Bronstein et al. [11]. A 300-mg amount of purified α-gliadin was suspended in 30 ml of 50 m*M* hydrochloric acid by stirring in a temperature-controlled (37°C) (Multitemp, LKB) chamber with a pH electrode. The suspension was incubated with 6 mg of pepsin (Spofa, Prague, Czechoslovakia) and was stopped after 120 min by changing the pH from 1.8 to 8.0 using 2 *M* sodium hydroxide solution. Calcium chloride was added up to a final concentration of 20 m*M*. The incubation then continued for 120 min with 6 mg of trypsin (Boehringer, Mannheim, F.R.G.) and subsequently 6 mg of pancreatine (Spofa) were added for an additional 120 min. During the 4 h the pH was automatically controlled by an Autoburette-TTT 60 (Radiometer, Copenhagen, Denmark). The proteolytic treatment was stopped by adding 0.1 *M* hydrochloric acid to pH 5.0 and the mixture was centrifuged for 15 min at 2000 *g*.

Ultrafiltration of PTP fragments

Fractionation of the PTP fragments of α-gliadin was carried out using an Amicon Model 52 concentration unit according to Dissanayake et al. [20] with minor modifications. Uncleaved material (subfraction $PTPa_1$) was removed on the first membrane, PM 30. The subfraction containing gliadin peptides ($PTPa_2$) was prepared by washing the ultrafiltrate followed by concentration on a YC 05 membrane. The residual subfraction ($PTPa_3$) contained the smallest peptides and free amino acids. The subfraction $PTPa_2$ was then lyophilized.

Analytical and preparative RP-HPLC

All separations were carried out using a Perkin-Elmer HPLC system with a Series-3B pump module controlled by a BASIC program and modified for automatic injection [21]. UV absorbance was monitored with a Model 85 UV LC detector with an 8-μl cuvette and the results were integrated by CIT-2 software on a Data Station 3600. Fractions (2 min) during preparative separation were collected in a SuperRac 2211 (LKB) fraction collector.

Solvents for HPLC separations were prepared from acetonitrile (Merck, Darmstadt, F.R.G.), trifluoroacetic acid (TFA) (Fluka, Buchs, Switzerland), ultra-pure water (Nanopur II, Wilhelm Werner, Berg Gladbach, F.R.G.) and after mixing were filtered through 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A.) and degassed by ultrasonication. Solvent A (0.1% TFA and 5% acetonitrile) and solvent B (0.1% TFA and 90% acetonitrile) were diluted fresh from a stock solution of 1% TFA (pH 2.5, adjusted with ammonia solution) stored at 4°C.

Analytical separation was performed on a stainless-steel column (300 mm$\times$4 mm I.D.) packed with 6-μm Separon SGX-C_{18} (Tessek, Prague, Czechoslovakia). A 300-μg amount of $PTPa_2$ subfraction was injected via a 20-μl loop on a 7125 Rheodyne sample injector and the separation was continued with a linear gradient from 3 to 35% acetonitrile at a flow-rate of 0.8 ml/min for 90 min, followed by 20-min gradient from 50 to 55% acetonitrile. The column was washed by elution for 8 min with 90% acetonitrile; the return and equilibration at 5% acetonitrile was carried out over 15 min.

The preparative separations were performed on a Knauer stainless-steel column (250 mm$\times$16 mm I.D.) packed with 10-μm Separon SGX-C_{18} (Tessek). A 100-mg amount of $PTPa_2$ subfraction in 1 ml of solvent A was applied to the column using a 1-ml loop and then eluted at a flow-rate of 2.5 ml/min. The gradient of acetonitrile lasted for 90 min from 5 to 35% and then for 45 min from 50 to 55%. The column was washed with 90% acetonitrile for 15 min; the return and equilibration at 5% acetonitrile was carried out over 35 min.

The columns were stabilized overnight at a flow-rate of 0.1 ml/min to achieve the optimal reproducibility.

Gel-permeation chromatography (GPC)

Molecular mass determination was carried out by GPC–HPLC separation on a UltroPac (LKB) column (300 mm$\times$7.5 mm I.D.) packed with TSK G2000 SW sorbent. The fractions $PTPa_{2\text{-}1}$ to $PTPa_{2\text{-}20}$ after RP–HPLC were lyophilized

and dissolved in 6 *M* guanidine hydrochloride. A 50-μl sample of each fraction was eluted at a flow-rate of 0.4 ml/min of 6 *M* guanidine hydrochloride (pH 4.95) [22] and detected at 279 nm. The standards for calibration were ovalbumin and ribonuclease (Pharmacia, Uppsala, Sweden), luliberin (luteinizing hormone–releasing hormone, LH–RH), melanostatin (melanocyte stimulating hormone releasing hormone–inhibiting factor, MIF) and insulin (Spofa). The GPC program (Perkin-Elmer) with a non-linear regression "point-to-point" was used for calibration and calculation of the molecular mass.

RESULTS

Pure α-gliadin was prepared by ion-exchange chromatography on sulfopropyl-Sephadex C-50, giving 800 mg of pure α-gliadin from 5 g of crude A-gliadin preparation. The eluates were evaluated by starch gel electrophoresis (Fig. 1). Peptide fragments were obtained by sequential proteolytic treatment with pepsin, trypsin and pancreatin followed by ultrafiltration on UM 10 and YC 05 membranes. The determination of peptides by using ninhydrin reagent [23] showed that the UM 10 membrane retained more than 90% of the fragments, and therefore we used the PM 30 membrane instead. This modification showed that 13% of the fragments were retained in subfraction $PTPa_1$ and 9.5% of the fragments were found in the ultrafiltrate through the YC 05 membrane (subfraction $PTPa_3$). The main product (subfraction $PTPa_2$) was used for the subsequent analysis.

The analysis of the $PTPa_2$ subfraction by RP-HPLC on a Separon SGX-C_{18} column with 0.1% TFA using acetonitrile gradient elution demonstrated at least

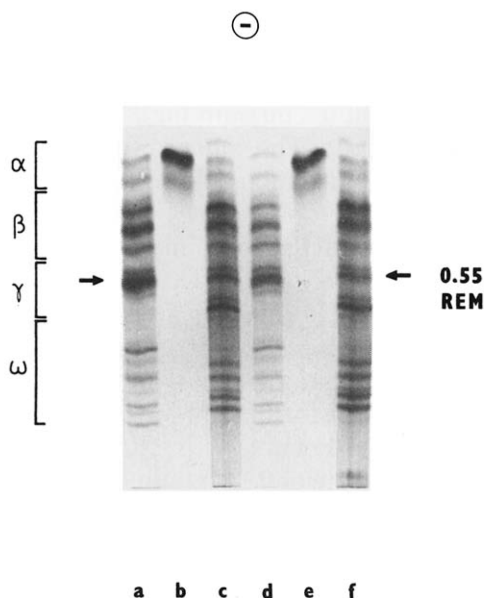

Fig. 1. Starch gel electrophoresis in aluminium lactate buffer (pH 3.1) of wheat extract (a,d), crude gliadin (c,f) and pure α-gliadin (b,e). The arrow indicates standard γ-gliadin band with a relative electrophoretic migration (REM) value of 0.55.

fifty peptide fragments (Fig. 2) and twenty main peaks were selected as $PTPa_{2\text{-}1}$ to $PTPa_{2\text{-}20}$. The reproducibility of the separation and the variation of the retention times during one week were up to 2.9%. The elution pattern of the $PTPa_2$ subfraction obtained by RP-HPLC was used as a standard for checking the reproducibility of the cleavage by proteolysis. By comparison of ten elution patterns during four years only quantitative changes were observed. The amount of a single fragment generated during proteolysis is therefore not constant and any two proteolyses can differ by up to 58% of the peak area for a single peak. These considerable differences for an individual peak area in terms of the whole separation changes represent only 6%.

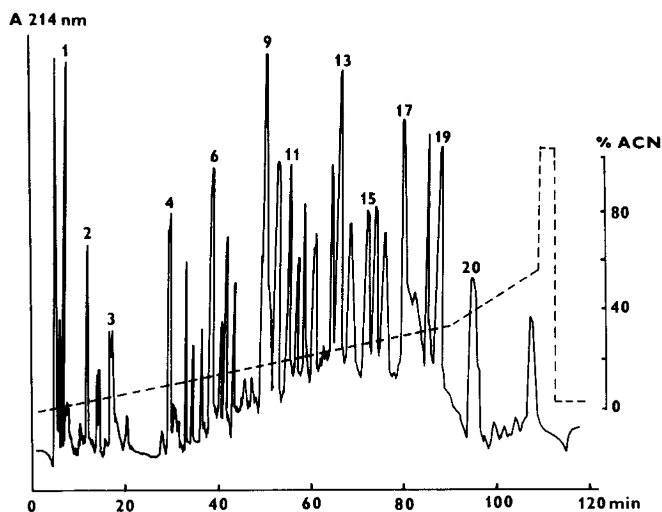

Fig. 2. Separation of 300 μg of $PTPa_2$ subfraction on a 300 mm×4 mm I.D. analytical column of Separon SGX-C_{18} (6 μm) using a gradient of acetonitrile (ACN) in 0.1% TFA.

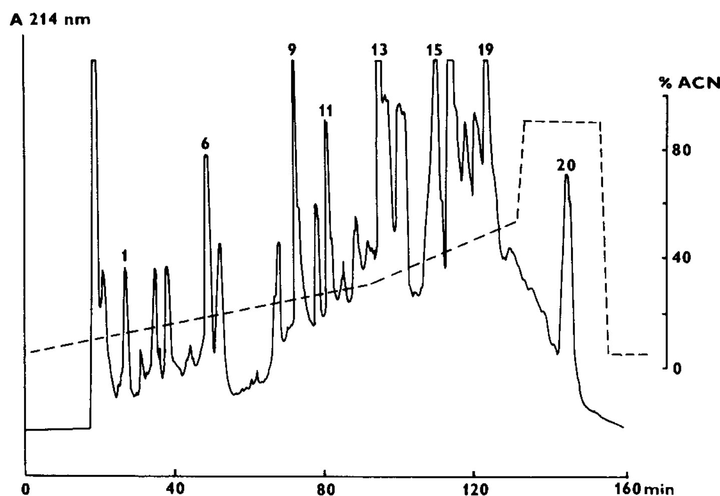

Fig. 3. Semi-preparative fractionation of 100 mg of $PTPa_2$ subfraction on a 250 mm×16 mm I.D. column of Separon SGX-C_{18} (10 μm) using a gradient of acetonitrile (ACN) in 0.1% TFA.

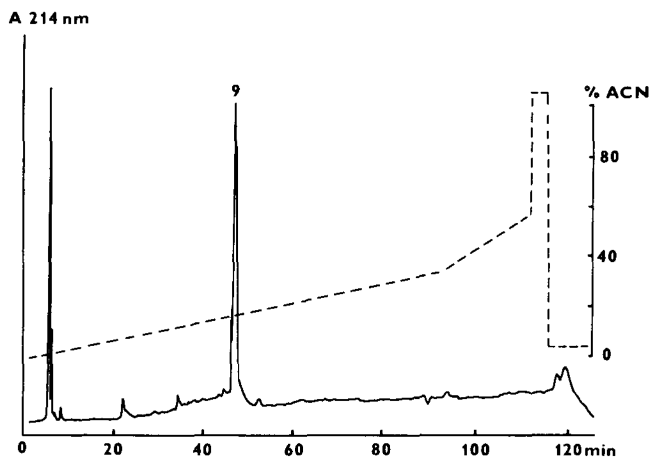

Fig. 4. Re-chromatography of purified fraction $PTPa_{2\text{-}9}$ by analytical separation of a Separon SGX-C_{18} column eluted with a gradient of acetonitrile (ACN) in 0.1% TFA.

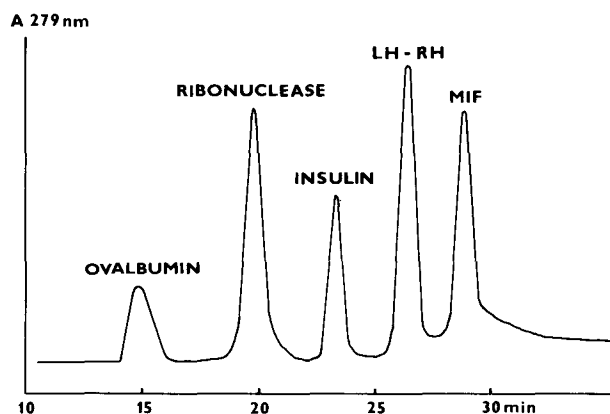

Fig. 5. Separation of calibration peptides by GPC on a 300 mm × 7.5 mm I.D. TSK G2000-SW column eluted with 6 *M* guanidine hydrochloride.

We converted the analytical separation to a semi-preparative scale for preparing large amounts of single fractions $PTPa_{2\text{-}1}$ to $PTPa_{2\text{-}20}$ on a 16 mm I.D. column packed with Separon SGX-C_{18}. We established the dependence of the separation efficiency on the flow-rate for calculation of the optimal linear mobile phase velocity to maintain similar conditions for both columns. The elution pattern (Fig. 3) shows a separation of 100 mg of the subfraction $PTPa_2$. The main peaks corresponding to peaks 1–20 in the analytical run were collected and lyophilized. Rechromatography on an analytical column was used to evaluate the preparative separation. Fig. 4 shows the chromatogram of the rechromatographed fraction $PTPa_{2\text{-}9}$. Its retention time of 45.03 min correlated with the value of 44.57 min given by the ninth peak during an analytical run.

The molecular mass and heterogeneity of each of fractions 1–20 were determined by GPC analysis on a TSK G2000 SW column after calibration with a mixture of five standard proteins (Fig. 5). Fig. 6 demonstrates the analysis of

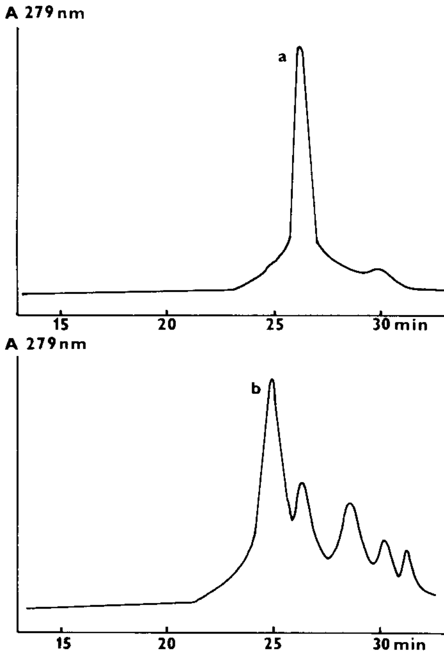

Fig. 6. GPC of purified fractions $PTPa_{2\text{-}9}$ (a) and $PTPa_{2\text{-}15}$ (b) on a TSK G2000-SW column eluted with 6 *M* guanidine hydrochloride.

TABLE I

CHARACTERIZATION OF FRACTIONS $PTPa_{2\text{-}1}$ TO $PTPa_{2\text{-}20}$ BY RP-HPLC ANALYSIS ON SEPARON SGX-C_{18} AND GPC TSK G2000-SW COLUMNS

Fraction No.	Retention time (min)	Molecular mass	Relative amount (%)
1	5.03	500	4.0
2	8.37	640	0.7
3	10.47	600	1.1
4	27.87	720	1.4
5	33.97	800	1.9
6	35.43	1500	2.1
7	40.10	790	2.3
8	42.33	730	1.3
9	44.57	1700	7.6
10	51.30	1100	1.2
11	53.50	1090	5.6
12	58.77	720	7.4
13	63.23	840	7.3
14	65.03	890	1.6
15	70.57	1530	2.7
16	73.47	2290	1.3
17	77.30	2420	4.1
18	83.67	1450	2.8
19	86.03	2130	8.5
20	92.47	1460	2.1

two fractions, $PTPa_{2\text{-}9}$ and $PTPa_{2\text{-}15}$, and shows that some fractions are homogeneous and other contain a heterogeneous mixture of up to six peptide fragments. The approximate relative molecular masses of fractions 1–20 are shown in Table I. The range for PTP fragments of α-gliadin was between 300 and 3000.

DISCUSSION

This study was concerned with the preparation of toxic fragments of gliadin for the elucidation of some etiopathogenetic factors in coeliac disease. The starting materials were crude gliadin or gluten, fractionated gliadin, i.e., A-gliadin prepared by centrifugation [24], or pure α-gliadin separated by ion-exchange chromatography [25]. Mühle et al. [26] found the material prepared by ion-exchange chromatography to be more suitable for further separations by comparing A-gliadin and α-gliadin. We chose a combination of both methods for this study and prepared pure α-gliadin by ion-exchange chromatography according to Charbonnier and Mossé [18] on SP-Sephadex C-50 from a crude A-gliadin. The chromatographic results were evaluated by using starch gel electrophoresis modified for a commercial LKB Multiphor system [19].

Isolated α-gliadin was treated with proteolytic enzymes. In this cleavage, more heterogeneous material consisting of dozens of peptide fragments with differences in molecular mass, hydrophobicity and surface properties have to be considered. The heterogeneity, mainly in molecular mass, depends on the time of cleavage and on the enzymes used. The smallest pure peptide with biological activity is that described by Offord et al. [27], Asp–Gln–Gln–Trp. Conversely, Wieser et al. [17] isolated a pure toxic peptide consisting of 53 amino acids with a molecular mass of 6162 using RP-HPLC. Jos et al. [28] reported the toxicity of a subfraction of PT digest with a molecular mass between 5000 and 10 000 and Cornell and Maxwell [29] demonstrated the toxicity of PTP digest of molecular mass 5000–6000. The uncleaved gliadin has a molecular mass of approximately 30 000–40 000. In order to mimic physiological conditions as closely as possible we used a sequential proteolysis with pepsin, trypsin and pancreatin similarly to other workers [11,29].

Ultrafiltration on a membrane is commonly used for subfractionation of the proteolytic digest. Using Amicon membranes, combinations of UM 10 and XM 100 [17,20] or PM 10 and UM 2 membranes [28] have been described. According to our results, the UM 10 membrane retains more than 90% of the material. Therefore, we used a combination of PM 30 and YC 05 membranes. The YC 05 membrane retains all fragments of molecular mass larger than approximately 300–500.

RP-HPLC on modified C_{18} sorbents has been used for gliadin analysis [13–15] and for isolating toxic peptides from gliadin [16,17]. Bietz [13] recommended elution with a 120-min gradient from 20 to 50% acetonitrile in 0.1% TFA, Jos et al. [16] described a gradient from 0 to 50% acetonitrile in 10 mM ammonium acetate and Wieser et al. [17] isolated the peptides with a gradient from 15 to 45% acetonitrile in 5 mM triethylammonium phosphate (TEAP). We tested a number of mobile phase additives, including ammonium acetate, phosphate, TFA

and heptafluorobutyric acid with different acetonitrile gradients. A 110-min gradient from 5 to 55% acetonitrile in 0.1% TFA was selected. TFA has the advantage over phosphate that it is easy to remove from eluted fractions during preparative separations. The elution patterns of the $PTPa_2$ subfraction can also be used for checking the reproducibility of the proteolytic treatment. In four years we have found only quantitative changes in the relative sizes of single peak areas, which represented a maximum of a 6% difference in terms of the whole separation.

We converted the analytical separation to the semi-preparative scale on a 16 mm I.D. column packed with a 10-μm sorbent and evaluated the linear mobile phase velocities for both columns. In a single run we are able to fractionate more than 100 mg of PTP-treated gliadin, which is 100 times more than reported previously [16,17].

The molecular mass of the peptides prepared by our method was found to be in range 300–3000. In all previous publications the molecular mass was evaluated by gel chromatography, sodium dodecyl sulphate polyacrylamide gel electrophoresis or by the technique used for preparation (i.e. ultrafiltration, etc.). We recommend the use of GPC on a TSK G2000 SW column. We found optimal linearity of calibration for the peptides of molecular mass between 400 and 30 000 using 6 *M* guanidine hydrochloride as described by Richter et al. [22]. The results of GPC analysis demonstrate not only the relative molecular mass but also the homogeneity of the fractions prepared.

REFERENCES

1 C. Alvey, C.M. Anderson and M. Freeman, Arch. Dis. Child., 32 (1957) 434.
2 D.D. Kasarda, Cereal Foods World, 23 (1978) 240.
3 W.Th.J.M. Hekkens, Dtsch. Z. Verdau. Stoffwechselkr., 43 (1983) 33.
4 D.D. Kasarda, Ann. Technol. Agric., 29 (1980) 151.
5 C.O. Farrelly, J. Kelly, W. Hekkens, B. Bradley, A. Thompson, C. Feighery and D.G. Weir, Br. Med. J., 286 (1983) 2007.
6 L. Charbonnier, J. Jos, J.F. Mougenot and J. Mossé, Reprod. Nutr. Dev., 20 (1980) 1369.
7 P.J. Ciclitira, D.J. Evans, E.S. Lennox, N.L.K. Fagg and R.H. Dowling, Clin. Sci., 62 (1982) 48p.
8 A.C. Frazer, R.F. Fletcher, C.A.A. Ross, B. Shaw, H.G. Sammons and R. Schneider, Lancet, ii (1959) 252.
9 C.O. Farrelly, W.T.J.M. Hekkens, C. Feighery and D.G. Weir, Scand. J. Gastroenterol., 18 (1983) 603.
10 H.J. Cornell and R.R.W. Townley, Clin. Chim. Acta, 43 (1973) 113.
11 H.D. Bronstein, L.J. Haffner and O.D. Kowlessar, Clin. Chim. Acta, 14 (1966) 141.
12 J. Jos, L. Charbonnier, J. Mossé, J.P. Olives, M.F. de Tand and J. Rey, Clin. Chim. Acta, 119 (1982) 263.
13 J.A. Bietz, J. Chromatogr., 255 (1983) 219.
14 T. Burnouf, J.A. Bietz, L.A. Cobb and J.S. Wall, C.R. Acad. Sci., 297 (1983) 377.
15 J.A. Bietz, Cereal Chem., 61 (1984) 124.
16 J. Jos, M.F. de Tand, F.A. Battandier, J.P. Boissel, Y. Popineau and H. Wajcman, Clin. Chim. Acta, 134 (1983) 189.
17 H. Wieser, H.D. Belitz, A. Ashkenazi and D. Idar, Z. Lebensm. Unters.-Forsch., 176 (1983) 85.
18 L. Charbonnier and J. Mossé, J. Sci. Food Agric., 31 (1980) 54.
19 P. Kocna, M. Holáková-Kočová and A. Šašek, Z. Lebensm. Unters.-Forsch., 177 (1983) 454.

20 A.S. Dissanayake, D.W. Jerrome, R.E. Offord, S.C. Truelove and R. Whitehead, Gut, 15 (1974) 931.
21 P. Kocna and B.J. Mittermüller, J. Chromatogr. Sci., 23 (1985) 132.
22 W.O. Richter, B. Jacob and P. Schwandt, Anal. Biochem., 133 (1983) 288.
23 H. Rosen, Arch. Biochem. Biophys., 67 (1957) 10.
24 J.E. Bernardin, D.D. Kasarda and D.K. Mecham, J. Biol. Chem., 242 (1967) 445.
25 A.L. Patey and D.J. Evans, J. Sci. Food Agric., 24 (1973) 1229.
26 W. Mühle, H. Remke, P. Walther and F. Müller, Acta Biol. Med. Ger., 38 (1979) 1543.
27 R.E. Offord, B.S. Anand, J. Piris and S.C. Truelove, in B. McNicholl (Editor), Perspectives in Coeliac Disease, MTP Press, Lancaster, 1978, p. 25.
28 J. Jos, L. Charbonnier, J.F. Mougenot, J. Mossé and J. Rey, in B. McNicholl (Editor), Perspectives in Coeliac Disease, MTP Press, Lancaster, 1978, p. 75.
29 H.J. Cornell and R.J. Maxwell, Clin. Chim. Acta, 123 (1982) 311.