Production of a panel of recombinant gliadins for the characterisation of T cell reactivity in coeliac disease

E H Arentz-Hansen, S N McAdam, Ø Molberg, C Kristiansen, L M Sollid

Abstract

Background/Aims—Coeliac disease is a chronic intestinal disorder most probably caused by an abnormal immune reaction to wheat gliadin. The identification of the HLA-DQ2 and HLA-DQ8 as the molecules responsible for the HLA association in coeliac disease strongly implicates a role for CD4 T cells in disease pathogenesis. Indeed, CD4 T cells specific for gliadin have been isolated from the small intestine of patients with coeliac disease. However, identification of T cell epitopes within gliadin has been hampered by the heterogeneous nature of the gliadin antigen. To aid the characterisation of gliadin T cell epitopes, multiple recombinant gliadins have been produced from a commercial Nordic wheat cultivar.

Methods—The α -gliadin and γ -gliadin genes were amplified by polymerase chain reaction from cDNA and genomic DNA, cloned into a pET expression vector, and sequenced. Genes encoding mature gliadins were expressed in *Escherichia coli* and tested for recognition by T cells.

Results—In total, 16 α -gliadin genes with complete open reading frames were sequenced. These genes encoded 11 distinct gliadin proteins, only one of which was found in the Swiss-Prot database. Expression of these gliadin genes produced a panel of recombinant α -gliadin proteins of purity suitable for use as an antigen for T cell stimulation.

Conclusion—This study provides an insight into the complexity of the gliadin antigen present in a wheat strain and has defined a panel of pure gliadin antigens that should prove invaluable for the future mapping of epitopes recognised by intestinal T cells in coeliac disease.

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Keywords: gliadin; coeliac disease; small intestine; protein expression; T lymphocyte; gluten

Coeliac disease is an inflammatory disorder of the small intestine characterised by atrophy of the villi and hyperplasia of crypt cells.^{1,2} The disease is precipitated in genetically susceptible people on ingestion of wheat gluten and the related proteins of rye and barley. Several lines of evidence indicate that the development of the disease is due to an abnormal immune response to gluten. The identification of HLA class II molecules DQ2 and DQ8 responsible

for the HLA association observed in coeliac disease^{3–8} implicates a role for coeliac disease T cells in disease pathogenesis. This is further supported by the demonstration that T cells extracted from intestinal biopsy specimens of patients with coeliac disease, but not of control patients, contain a population that are coeliac disease positive and $\alpha\beta$ T cell receptor positive and specific for gliadin. These gut derived T cells are conspicuous in that the vast majority recognise gliadin peptides when presented by DQ2 or DQ8.^{9–11} Gluten comprises numerous related proteins which together provide a nitrogen store for the germinating seed. On the basis of their solubility in aqueous alcohol, gluten can be separated into the gliadins (soluble) and the glutenins (insoluble). The gliadins are monomer molecules which can be further divided into three classes on the basis of sequence (α -, γ -, and ω -gliadins).¹² Gliadins in each class differ slightly in sequence,¹³ and in a single wheat variety at least 45 different gliadins have been identified at the protein level.¹⁴ The glutenins consist of large polymeric structures that are formed as the result of intermolecular disulphide bonds. Most T cells isolated from biopsy specimens challenged with gluten appear to recognise the alcohol soluble gliadin fraction.⁹ Interestingly, this recognition of gliadin by intestinal T cells is dependent on deamidation, a modification known to be promoted in the acidic environment during pepsin digestion. However, deamidation is most probably mediated in vivo in a specific fashion by the enzyme tissue transglutaminase (tTG).¹⁵ This enzyme mediated deamidation introduces negative charges into gliadin and increases the binding affinity of gliadin peptides for DQ2. A better understanding of the processes that lead to the modification and T cell recognition of gliadin peptides, and which ultimately cause disease, requires that the intestinal T cell response to the gliadin antigen be characterised at a molecular level. Identification of T cell epitopes in gliadin has been complicated by the enormous micro-heterogeneity of the complex gliadin antigen and the difficulty in purifying antigens with a defined sequence. To aid the characterisation of T cell epitopes, we have cloned, sequenced, and expressed a panel of gliadins in *Escherichia coli* and tested them for their ability to stimulate

Institute of Immunology, Rikshospitalet, University of Oslo, Norway

E H Arentz-Hansen
S N McAdam
Ø Molberg
C Kristiansen
L M Sollid

Correspondence to:
Dr E H Arentz-Hansen,
Institute of Immunology,
Rikshospitalet, 0027 Oslo,
Norway.

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Abbreviations used in this paper: PCR, polymerase chain reaction; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; tTG, tissue transglutaminase.

gut derived T cell clones of patients with coeliac disease.

Materials and methods

GLIADIN GENE

The plasmid containing the γ -gliadin gene pW1621 was a gift from A Rafalski (DuPont Agricultural Biotechnology, Wilmington, Delaware, USA).

ISOLATION OF DNA AND mRNA AND SYNTHESIS OF cDNA

The Nordic autumn wheat strain Mjoelner was the source of genomic DNA and mRNA. The wheat endosperm was harvested 30 days after flowering. Genomic DNA was phenol extracted from the endosperm or blades. mRNA was isolated from the endosperm using the Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway). For cDNA synthesis, RNA was eluted from the beads and added to a mixture containing 1 mM dNTP (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK), 1 μ M oligo(dT)₁₅ primer (Promega, Madison, Wisconsin, USA), 20 mM dithiothreitol (Gibco-BRL, Paisley, Scotland, UK), 20 U RNasin (Promega), and 200 U Moloney murine leukaemia virus (Gibco-BRL) and incubated for one hour at 37°C. The enzymes were inactivated by heating at 65°C for five minutes.

AMPLIFICATION AND CLONING OF GLIADIN GENES

Oligonucleotide primers (Eurogentec, Herstal, Belgium) were designed for amplification of gliadin genes coding for mature proteins without leader sequences. The α -gliadin specific primers had the sequences: sense primer 5' GCT ATG GAT CCA TAT GGT TAG AGT TCC AGT GCC and antisense primer 5' GCA TCA AGC TTC ATC GAT AGT TAG TAC CGA AGA TGC C. The γ -gliadin specific primers had the sequences: sense primer 5' GCT ATG GAT CCA TAT GAA TAT CCA GGT CGA CCC and antisense primer 5' GCA TCA AGC TTC ATC GAT ATT GGC CAC CAA TGC CCG C. In addition to start and stop codons, *Hind*III and *Nde*I restriction sites were included in the primer sequences for cloning and expression purposes.

For polymerase chain reaction (PCR) amplification, about 100 ng genomic DNA or cDNA was used in a reaction mixture containing 1 mM each dNTP (Amersham Pharmacia Biotech), 200 nM each primer, 2 mM MgCl₂, 1.25 U *Taq* DNA polymerase (Promega), and 0.008 plaque forming units polymerase (Stratagene, La Jolla, California, USA). The denaturation, annealing, and polymerisation temperatures for the first five cycles were 94°C, 45°C, and 72°C respectively. In the following 30 cycles the annealing temperature was increased to 55°C. Purified PCR fragments were cloned into the *Nde*I and *Hind*III site of the pET17xb vector according to the instructions in the manufacturer's manual (Novagen, Madison, Wisconsin, USA). The ligation mixture was used to transform competent Nova Blue cells (Novagen), and recombinant plasmids were isolated using Wizard Plus SV Minipreps (Promega).

DNA SEQUENCING

Cycle sequencing of gliadin clones was performed on PCR products amplified with T7 vector specific primers using the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Pharmacia Biotech) according to the manufacturer's manual. Two vector specific sequencing primers were used for both α -gliadin and γ -gliadin: T7 promoter primer, 5' TAA TAC GAC TCA CTA TAG GG; T7 terminator primer, 5' GCT AGT TAT TGC TCA GCG G. The internal α -gliadin specific primers were: (a) 5' GCA TGG ATG TTG TAT T, (b) 5' CTG CAA TAC AAC ATC C, (c) 5' GCA GGG ATG TTG TCT T, (d) 5' TTG CAA GAC AAC ATC C, (e) 5' TGC TGA CAA CAC AAT T. The internal γ -gliadin specific primers were: (a) 5' ATT CTT GCA TGG GTT CA; (b) 5' TGA ACC CAT GCA AGA AT. Sequencing products were run on an ABI Prism 377XL DNA sequencer (Perkin Elmer, Norwalk, Connecticut, USA).

PRODUCTION OF RECOMBINANT GLIADINS IN *E. COLI*

Plasmids containing gliadin inserts of appropriate lengths were transformed into competent BL21(DE3)pLysS cells (Novagen) containing a single copy of the T7 polymerase gene under the control of the inducible lac UV5 promoter. Freshly plated single colonies were grown in 1 litre cultures of Luria-Bertani medium containing 100 μ g/ml carbenicillin and 34 μ g/ml chloramphenicol at 37°C. At a culture density of $A_{600} = 0.6$, isopropyl β -D-thiogalactoside (Sigma, St Louis, Missouri, USA; 0.4 mM) was added and the cultures were incubated for a further 18 hours. At harvesting, cell pellets obtained by centrifugation (15 minutes at 650 g and 30 minutes at 2520 g) were resuspended in preheated (60°C) 70% ethanol and incubated at 60°C for two hours. Bacterial cell debris was then removed by centrifugation (30 minutes at 14 500 g), and two volumes of 1.5 M NaCl was added to the supernatant to precipitate the gliadin proteins. The solution was incubated at 4°C overnight to allow complete precipitation of the gliadins. The precipitate was collected by centrifugation (30 minutes at 14 500 g), rinsed briefly with distilled water, and dissolved in 8 M urea/0.4 M NH₄HCO₃. The proteins were analysed using one dimensional sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) with 12% gels and staining with Brilliant Blue R250 (Sigma). Protein was determined with a BCA protein assay (Pierce, Rockford, Illinois, USA) using commercial gliadin (Sigma) as a standard.

PREPARATION OF RECOMBINANT GLIADINS FOR T CELL ASSAYS

The solubilised gliadins were dialysed against 0.1 M NH₄HCO₃ overnight to remove the urea. Gliadin proteins are hardly soluble in physiological salt solutions, but can be made soluble through limited enzymatic proteolysis and thereby become suitable as antigens in T cell assays. The recombinant gliadins used in T

cell assays were therefore digested with either α -chymotrypsin (C-4129; Sigma; 1:100, w/w) at 37°C in 0.1 M NH_4HCO_3 until dissolved or pepsin (P7012; Sigma; 1:100, w/w) at 37°C in 0.1 M acetic acid, pH 1.8, for three hours. The chymotrypsin digested recombinant gliadins (125 $\mu\text{g}/\text{ml}$) were incubated with 250 $\mu\text{g}/\text{ml}$ guinea pig tTG (T-5398; Sigma) at 37°C for two hours in phosphate buffered saline with 0.8 mM CaCl_2 whereas the pepsin treated recombinant gliadins were heat treated in the digestion buffer at 98°C for two hours before addition to T cell assays.

T CELL ASSAY

The following gut derived DQ2 restricted T cell clones of patients with coeliac disease were used: CD 380 E-27, CD 387 E-34, CD 412 R-3,¹⁶ CD 370 R-2.3, and CD 412 R-5.32 (Ø Molberg, unpublished). The last two clones were cloned using a protocol identical with that for the published clones. In brief, gliadin specific T cell lines were established from small intestinal biopsy specimens challenged in vitro with a peptic-tryptic digest of gliadin. The gliadin specific T cell lines were cloned at limiting dilution to establish gliadin specific T cell clones.

T cells (5×10^4) were added to 5×10^4 antigen presenting cells (HLA matched allogenic Epstein Barr virus transformed B lymphoblastoid cell lines irradiated with 80 Gy) that had been incubated for 16–20 hours with either untreated or tTG treated recombinant gliadins (50 $\mu\text{g}/\text{ml}$) in a volume of 100 μl RPMI 1640 (Life Technologies, Rockville, Maryland, USA) supplemented with 15% pooled and heat inactivated human serum. The assay was performed in 96-well U bottom plates (Costar, Cambridge, Massachusetts, USA), and T cell proliferation was measured as [^3H]thymidine incorporation 48–72 hours after the antigenic stimulation. Activation of the 60.6 T cell transfectant¹⁵ was measured as interleukin-2 release after an 18 hour incubation of 2.5×10^4 transfectant cells with 5×10^4 non-irradiated antigen pulsed DQ2 positive B lymphoblastoid cells. Murine interleukin-2 was quantified by time resolved fluorometry using Delfia reagents (Wallac, Turku, Finland) and the rat anti-murine interleukin-2 antibodies JES6-IA12 and JES6-5H4 (PharMingen, San Diego, California, USA).

Figure 1 Recognition of pepsin treated pW1621 recombinant gliadin by the T cell transfectant 60.6. Pepsin-tryptic treated crude gliadin from Sigma (PT gliadin) was used as positive control. T cell activation was measured as interleukin-2 release and is given as arbitrary europium (Eu) counts. T+APC, T cells plus antigen presenting cells.

Results

ESTABLISHMENT OF THE EXPRESSION SYSTEM

As yet only one gliadin epitope that is recognised by gut derived DQ2 restricted T cells of patients with coeliac disease has been described.¹⁷ This γ -gliadin epitope is created after deamidation of a critical glutamine residue by either heat treatment in an acidic environment¹⁷ or enzymatically by tTG.¹⁵ To validate the suitability of the selected *E coli* expression system for the characterisation of T cell epitopes, we cloned a γ -gliadin gene (pW1621) that contains the published epitope into the pET17xb expression vector and expressed it in BL21(DE3)pLysS cells. Sequencing of plasmids obtained from PCR amplification of the pW1621 plasmid with subsequent cloning of the PCR product into the pET vector identified several positions that diverged from the published sequence (data not shown). In two out of seven pW1621 clones, single substitutions were found that are likely to represent misincorporation of nucleotides by the DNA polymerase. This allows a rough estimate of the PCR error rate in this system to be calculated as 1 in 2775. In contrast with the PCR introduced errors, seven substitutions were found in all clones and are probably due to sequencing errors reported in the sequence published in 1987. Notably, these substitutions cause a change in five amino acids from the deduced protein sequence—that is, residues 192 S→C, 255 I→M, 256 D→H, 264 H→Q, 265 E→Q; numbering relates to protein with leader sequence. Attempts to use bacterial lysates as T cell antigen failed because the T cell clones reacted non-specifically with bacterial material (data not shown). It was therefore necessary to purify the gliadin proteins from the bacterial lysates. For the purification, we adapted a protocol of A Tatham (personal communication) in which the gliadins are extracted with aqueous alcohol and precipitated with NaCl. From lysates of bacteria expressing the pW1621 gene, we obtained a relatively pure protein of apparent mass 36 kDa as assessed by SDS/PAGE (data not shown). This recombinant γ -gliadin protein was then digested with pepsin and acid/heat treated and tested for its ability to stimulate a T cell transfectant specific for this epitope.¹⁵ Reassuringly, the recombinant protein efficiently stimulated this transfectant (fig 1).

AMPLIFICATION, CLONING, AND SEQUENCING OF GLIADIN GENES

We next wanted to express a panel of different gliadin genes coding for proteins to which the Norwegian population is exposed. Thus cDNA and genomic DNA were isolated from a wheat strain (Mjoelner) commonly grown in Norway. The α -gliadin genes were amplified from both cDNA and genomic DNA whereas the γ -gliadin genes were amplified from genomic DNA only. Altogether, 16 independent α -gliadin clones were sequenced. Eleven unique sequences were obtained. Notably, only one of the deduced protein sequences (α -2) identically matched a previous entry in the Swiss-Prot data bank (accession number

Figure 2 Amino acid sequence alignment of the α -gliadin clones. The EMBL accession numbers of the DNA sequence and the clone names are indicated. A consensus amino acid sequence is given above the alignment. The N-terminal M and the C-terminal Y and R are non-gliadin sequences that are introduced as part of the expression vector. The sequences of the six N-terminal residues and the eight C-terminal residues are determined by the primers used for the PCR amplification.

P18573¹⁸) (fig 2). Sequencing of the first 400 bases on the 5' end of 15 independent clones obtained from a PCR with γ -gliadin specific primers gave seven sequences unrelated to gliadin, and eight gliadin sequences. Complete sequencing of two of these clones identified one mature γ -gliadin not previously described in the database (GenBank accession number AJ133613) and one pseudogene.

Figure 3 SDS/PAGE of the recombinant gliadins, before and after purification. Lane 1, molecular mass markers; lane 2, bacterial lysate from batch 1; lane 3, purified recombinant gliadins from batch 1; lane 4, lysate from bacteria expressing a single gliadin (α -11, not present in batch 1); lane 5, purified α -11. The bacterial lysates were harvested 18 hours after induction with isopropyl β -D-thiogalactoside.

EXPRESSION AND T CELL RECOGNITION OF BATCHES OF RECOMBINANT α -GLIADINS

Two batches, each containing nine randomly picked α -gliadin clones, were produced in 1 litre cultures. SDS/PAGE analysis of the purified gliadins disclosed bands with apparent masses of 36–40 kDa (fig 3). After chymotrypsin digestion, the complex gliadins either untreated or treated with tTG, were tested for recognition by four different gliadin specific gut derived T cell clones in proliferation assays. Both batches contained material that was recognised by all the T cell clones, but only after treatment with tTG (fig 4).

EXPRESSION AND T CELL RECOGNITION OF SINGLE RECOMBINANT α -GLIADINS

The nine gliadins from batch 1 were then expressed in individual 1 litre cultures. Both the apparent molecular mass (data not shown) and the yield for each of the gliadins (table 1) varied between the different clones. DNA sequencing of the seven clones that produced protein showed five unique sequences, α -1, α -2, α -3, α -4, and α -5 (fig 2 and table 1). These five gliadins were then tested for their ability to stimulate four different T cell clones, three of which were previously shown to stimulate batch 1. Four of the five recombinant gliadins failed to stimulate any of the T cell clones. However, one recombinant gliadin (α -2) stimulated all of the T cell clones. Stimulation of the T cell clones by this recombinant was entirely dependent on treatment with tTG (fig 5).

Discussion

We have cloned a panel of gliadin genes with distinct sequences from a wheat strain used for food production and expressed their respective

Figure 4 Recognition of batches of chymotrypsin digested and tissue transglutaminase (tTG) treated recombinant α -gliadins by the gut derived DQ2 restricted T cell clone (TCC) CD 412 R-5.32. Batches 1 and 2 each contain nine α -gliadin clones. Crude gliadin prepared from the wheat strain Kadett was used as a positive control. Similar data were obtained from the other T cell clones tested—that is, CD 380 E-27, CD 387 E-34, and CD 370 R2.3. T+APC, T cells plus antigen presenting cells.

Table 1 Protein-expressing clones from batch 1

α -gliadin clone	Protein yield (mg/l culture)	Deduced molecular mass (kDa)
α -1*	28.3	30.6
α -2*	19.6	33.7
α -3	21.0	32.2
α -4	24.7	32.2
α -5	10.7	32.5

*Clones present twice in this batch.

proteins in *E. coli*. The gliadins were easy to produce and purify. Furthermore, a recombinant gliadin was shown to efficiently stimulate antigen specific T cells in in vitro assays. The availability of recombinant gliadins with defined sequences should facilitate the identification of T cell epitopes that are involved in the development of coeliac disease.

T cells are likely to be involved in the pathogenesis of coeliac disease. This notion is supported by the strong disease association with DQ2 and DQ8 and the presence of gliadin specific and DQ2 or DQ8 restricted T cells in the small intestinal mucosa of patients with coeliac disease. T cells recognise antigens in

Figure 5 Recognition of one of the recombinant gliadin subtypes (α -2) after chymotrypsin digestion and tissue transglutaminase (tTG) treatment of the T cell clone (TCC) CD 412 R-5.32. Similar data were obtained from the other T cell clones tested—that is, CD 380 E-27, CD 412 R-3, and CD 370 R2.3. T+APC, T cells plus antigen presenting cells.

the form of peptide fragments (10–15 amino acids) bound to HLA molecules. Small variations in peptide antigen sequence may affect T cell recognition.¹⁹ Notably, gliadin is not a simple protein but is, in fact, a complex mixture of distinct proteins that differ by minor variations in amino acid sequence. The sequence variation found between these proteins is likely to be important for their recognition by T cells.

The isolation of a single species of a gliadin protein from wheat flour by biochemical methods is difficult and laborious,^{20, 21} and there is always a danger that the isolated protein actually comprises several different species with indistinguishable physicochemical properties. Furthermore, gliadins have stretches of repetitive glutamines and high contents of proline which pose particular problems for N- and C-terminal amino acid sequencing. Moreover, gliadin peptides are difficult to sequence by tandem mass spectrometry because they often fragment poorly. The challenge to identify the sequence of epitopes is further compounded by the fact that most gliadin T cell epitopes recognised by gut T cells contain deamidated glutamines.¹⁷ These reasons motivated us to express recombinant proteins. The knowledge of the exact sequence of the whole protein provides a scaffold, which enables efficient sequence identification from limited mass spectrometry data. Thus the use of recombinants should allow the information of the sequence of stimulatory and non-stimulatory gliadins to be combined with the full power of mass spectrometry for sequence analysis to identify T cell epitopes.

The cloning and expression of a γ -gliadin gene (pW1621) containing a defined epitope allowed the potential of a recombinant expression system to be evaluated. Protein extracted with aqueous ethanol and precipitated by the addition of salt from bacteria expressing this γ -gliadin gene migrated as a single band of the expected size. Moreover, the γ -gliadin was recognised by a T cell transfectant specific for the defined epitope,^{9, 15} indicating that this approach of expressing recombinant gliadins could be useful for the mapping of gliadin epitopes.

Several new α -gliadin sequences that were not present in the Swiss-Prot database were obtained in our study. This may indicate that there exist many more α -gliadin variants than those currently deposited in this database. The high number of distinct sequences may also reflect errors created during the PCR amplification of the genes. Two types of PCR errors can be envisaged. One is the misincorporation of nucleotides. However, based on the error rate found in a similar PCR with a γ -gliadin gene, this is likely to account for only a small part of the variability. A second error during the PCR could arise as partial products generated in one amplification cycle serve as primers in a subsequent cycle for distinct templates, thereby creating genes with shuffled sequences. To what extent this actually has taken place is difficult to ascertain. However, this should not be a serious flaw to the objective of using recombinant gliadins for characterisation of T

cell epitopes, as the latter consist of short peptide fragments of 10–15 amino acids for which the coding sequences are likely to be maintained.

The sequencing of 400 bases at the 5' end of 15 PCR clones amplified using the γ -gliadin specific primers identified seven sequences that were unrelated to gliadin while the remaining eight clones contained sequences encoding γ -gliadins. Full length sequencing of two of these clones showed one pseudogene and one full length gliadin which was successfully expressed.

The approach of expressing recombinant gliadins to characterise T cell epitopes has a limitation if a restricted number of distinct genes are represented. It is notable that the consensus sequence obtained from the α -gliadin clones was remarkably similar to that of A-gliadin,²² with a discrepancy only in six positions. The A-gliadin sequence is essentially a biochemical "consensus" sequence obtained by amino acid sequencing of gliadin peptides. This suggests that the α -gliadin genes that we have described are broadly representative of the gliadin expressed naturally. This notion is also supported by a sequence cluster analysis of the deduced amino acid sequence of the genes cloned by us and the α -gliadin genes present in the Swiss-Prot database. The sequences identified by us were evenly distributed throughout the dendrogram (data not shown), indicating that the conditions we used for amplification and cloning of our α -gliadin genes did not result in the preferential cloning of a limited subset of gliadin genes.

We started by expressing nine different α -gliadin clones in two separate batches to obtain a broad representation of distinct sequences. As both batches were recognised in proliferation assays by four different T cell clones, we individually expressed the clones from one of them. Only seven out of nine α -gliadin clones yielded mature α -gliadin protein, and the amount of α -gliadin produced differed considerably among the different clones. This is most probably due to variation in the expression system, especially in the purification process of the recombinant gliadins. By testing each of the individual α -gliadins for recognition by T cell clones, we found that only one of the α -gliadin subtypes was recognised and that this was recognised by all four T cell clones tested. This indicates that most of the α -gliadin subtypes do not contain epitopes recognised by these T cells.

Our study shows that gliadins can be easily produced by recombinant DNA technology. Multiple proteins need to be expressed for T cell epitope characterisation, as T cells discriminate between distinct gliadins. The extra workload of expressing numerous gliadins is compensated for by a purification step, which is simple and efficient compared with the commonly used protocols for recombinant proteins that employ tags and affinity purification. This panel of recombinant gliadins, together with

mass spectrometry analysis, should allow the efficient identification of T cell epitopes in gliadins.

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