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Genomic, biochemical and modelling analyses of asparagine synthetases from wheat

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Author contribution statement

HX. Molecular cloning, expression and biochemical analysis of wheat asparagine synthetases
TYC. Modelling
SJP. Statistical analyses
SR. Genomic analyses
RG. Nucleotide sequence analysis of wheat asparagine synthetases
JH. Joint project leader
MH. Modelling
DG. Modelling
NGH. Joint project leader and lead author

Keywords

wheat, asparagine synthetase, Acrylamide, Food Safety, enzyme activity, mathematical modelling

Abstract

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Asparagine synthetase activity in cereals has become an important issue with the discovery that free asparagine concentration determines the potential for formation of acrylamide, a probably carcinogenic processing contaminant, in baked cereal products. Asparagine synthetase catalyses the ATP-dependent transfer of the amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine. Here, asparagine synthetase-encoding polymerase chain reaction products were amplified from wheat (Triticum aestivum) cv. Spark cDNA. The encoded proteins were assigned the names TaASN1, TaASN2 and TaASN3 on the basis of comparisons with other wheat and cereal asparagine synthetases. Although very similar to each other they differed slightly in size, with molecular masses of 65.49, 65.06 and 66.24 kDa, respectively. Chromosomal positions and scaffold references were established for TaASN1, TaASN2 and TaASN3, and a fourth, more recently identified gene, TaASN4. TaASN1, TaASN2 and TaASN4 were all found to be single copy genes, located on Chromosomes 5, 3 and 4, respectively, of each genome (A, B and D), although variety Chinese Spring lacked a TaASN2 gene in the B genome. Two copies of TaASN3 were found on Chromosome 1 of each genome, and these were given the names TaASN3.1 and TaASN3.2. The TaASN1, TaASN2 and TaASN3 PCR products were heterologously-expressed in Escherichia coli (TaASN4 was not investigated in this part of the study). Western blot analysis identified two monoclonal antibodies that recognised the three proteins, but did not distinguish between them, despite being raised to epitopes SKKPRMIEVAAP and GGSNKPGVMNTV in the variable C-terminal regions of the proteins. The heterologously-expressed TaASN1 and TaASN2 proteins were found to be active asparagine synthetases, producing asparagine and glutamate from glutamine and aspartate. The asparagine synthetase reaction was modelled using SNOOPY® software and information from the BRENDA database to generate differential equations to describe the reaction stages, based on mass action kinetics. Experimental data from the reactions catalysed by TaASN1 and TaASN2 were entered into the model using Copasi, enabling values to be determined for kinetic parameters. Both the reaction data and the modelling showed that the enzymes continued to produce glutamate even when the synthesis of asparagine had ceased due

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

Genomic, biochemical and modelling analyses of asparagine synthetases from wheat

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ABSTRACT

Asparagine synthetase activity in cereals has become an important issue with the discovery that free asparagine concentration determines the potential for formation of acrylamide, a probably carcinogenic processing contaminant, in baked cereal products. Asparagine synthetase catalyses the ATP-dependent transfer of the amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine. Here, asparagine synthetase-encoding polymerase chain reaction products were amplified from wheat (*Triticum aestivum*) cv. Spark cDNA. The encoded proteins were assigned the names TaASN1, TaASN2 and TaASN3 on the basis of comparisons with other wheat and cereal asparagine synthetases. Although very similar to each other they differed slightly in size, with molecular masses of 65.49, 65.06 and 66.24 kDa, respectively. Chromosomal positions and scaffold references were established for *TaASN1*, *TaASN2* and *TaASN3*, and a fourth, more recently identified gene, *TaASN4*. *TaASN1*, *TaASN2* and *TaASN4* were all found to be single copy genes, located on Chromosomes 5, 3 and 4, respectively, of each genome (A, B and D), although variety Chinese Spring lacked a *TaASN2* gene in the B genome. Two copies of *TaASN3* were found on Chromosome 1 of each genome, and these were given the names *TaASN3.1* and *TaASN3.2*. The TaASN1, TaASN2 and TaASN3 PCR products were heterologously-expressed in *Escherichia coli* (TaASN4 was not investigated in this part of the study). Western blot analysis identified two monoclonal antibodies that recognised the three proteins, but did not distinguish between them, despite being raised to epitopes SKKPRMIEVAAP and GGSNKPGVMNTV in the variable C-terminal regions of the proteins. The heterologously-expressed TaASN1 and TaASN2 proteins were found to be active asparagine synthetases, producing asparagine and glutamate from glutamine and aspartate. The asparagine synthetase reaction was modelled using SNOOPY® software and information from the BRENDA database to generate differential equations to describe the reaction stages, based on mass action kinetics. Experimental data from the reactions catalysed by TaASN1 and TaASN2 were entered into the model using Copasi, enabling values to be determined for kinetic parameters. Both the reaction data and the modelling showed that the enzymes continued to produce glutamate even when the synthesis of asparagine had ceased due to a lack of aspartate.

Keywords: wheat, asparagine synthetase, acrylamide, food safety, enzyme activity, mathematical modelling

INTRODUCTION

Asparagine is an important nitrogen storage and transport molecule in many plant species due to its relatively high nitrogen to carbon ratio (2:4, compared with 2:5 for glutamine, 1:5 for glutamic acid and 1:4 for aspartic acid, for example) and its relative chemical inertia (Lea *et al*., 2007). It accumulates in its free (non-protein) form in response to a range of abiotic and biotic stresses, as well as during normal physiological processes such as seed germination (Lea *et al*., 2007). In wheat grain it accumulates to very high levels in response to sulphur deficiency (Muttucumaru *et al*., 2006; Granvogl *et al*., 2007; Curtis *et al*., 2009; 2018) and poor disease control (Curtis *et al*. 2016). There are also large differences in the free asparagine concentration of grain from different wheat varieties (Curtis *et al*., 2018). Understanding the mechanisms that control free asparagine accumulation is important for improving crop yield and stress resistance. However, more pressingly, it also has implications for food safety because free asparagine is a precursor for acrylamide formation (reviewed by Curtis *et al*., 2014; Halford *et al*., 2012).

Acrylamide is a processing contaminant that forms during high-temperature cooking and processing, particularly as a result of frying, roasting and baking. It is classed as a probable (Group 2a) carcinogen by the International Agency for Research on Cancer (1994) and has reproductive and neurotoxicological effects at high doses (Friedman, 2003). The European Food Safety Authority (EFSA) Expert Panel on Contaminants in the Food Chain (CONTAM) stated in its 2015 report that the margins of exposure for acrylamide indicate a concern for neoplastic effects (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2015). The European Commission issued 'Indicative Values' for the presence of acrylamide in food in 2011, based on results reported to EFSA (European Food Safety Authority, 2011), and reduced them for many product types in 2013 (European Commission, 2013). If a product is found to exceed the Indicative Value, the relevant food safety authority should take action to ensure that the manufacturer addresses the problem. Furthermore, the European Commission has just approved strengthened risk management measures including compulsory Codes of Practice and the renaming of Indicative Values as Benchmark Levels, with reduced Benchmark Levels for many products (European Commission, 2017). The proposals also include a specific reference to the setting of mandatory Maximum Levels for acrylamide in certain foods, stating that this should be considered following the adoption of the new regulations. The proposals will come before the European Parliament and European Council in 2017 and could be in force by early 2018.

The predominant route for the formation of acrylamide is *via* a Strecker-type degradation of free asparagine by highly reactive carbonyl compounds produced within the Maillard reaction (Mottram *et al*., 2002; Stadler *et al*., 2002; Zyzak *et al*., 2003), and free asparagine concentration is the main determinant of acrylamide-forming potential in cereal grains (Muttucumaru *et al*., 2006; Granvogl *et al*., 2007; Curtis *et al*., 2009; 2010; Postles *et al*., 2013). This has re-invigorated interest in the enzymes involved in asparagine synthesis and breakdown, and other metabolic pathways that could impact on free asparagine concentrations. Asparagine synthesis is catalysed by the enzyme asparagine synthetase, and occurs by the ATP-dependent transfer of the amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine, and. Two asparagine synthetase genes were cloned from wheat (*Triticum aestivum*) by Wang *et al*. (2005) and called *TaASN1* and *TaASN2*. *TaASN1* expression in seedlings was shown to be up-regulated by treatment with abscisic acid (ABA), and by salt and osmotic stress (Wang *et al*. 2005). Subsequently, its expression in leaves was shown to be induced by sulphur deficiency, but to be greatly reduced when a general control

non-derepressible-2-type protein kinase, TaGCN2, was over-expressed (Byrne *et al*., 2012). In 2016, two additional genes, *TaASN3* and *TaASN4*, were identified (Gao *et al*., 2016), although *TaASN4* was only discovered from wheat genome data and has not yet been cloned or characterized. The expression of *TaASN1-3* was studied in different tissues and in response to nutrition (Gao *et al*., 2016). Notably, the expression of *TaASN2* in the embryo and endosperm during mid to late grain development was shown to be the highest of any of the genes in any tissue, although *TaASN1* was most responsive to sulphur supply.

Maize (*Zea mays*) and barley (*Hordeum vulgare*) also have four differentially-expressed asparagine synthetase genes (Todd *et al*., 2008; Avila-Ospina *et al*., 2015), suggesting that this is typical of the cereals. However, a full picture of the role of the different asparagine synthetases will only emerge when the kinetic parameters of the enzymes have been measured. This is problematic because asparagine synthetase activity in plant tissues is difficult to purify and measure (Joy *et al*.,1983; Snapp and Vance, 1986; Kudiyarova *et al*., 2013), probably because of the presence of asparaginase activities and natural inhibitors (Rognes, 1980). There has also been a scarcity of antibodies for immunological analysis of purified or expressed proteins. However, the enzymes encoded by three of the maize genes have been analysed after heterologous expression in *Escherichia coli* and been shown to have significant differences in kinetic properties (Duff *et al*., 2011). The aim of this study was to characterize the wheat asparagine synthetase gene family and to compare the enzymes encoded by *TaASN1* and *TaASN2*, the two genes that are most highly expressed in the grain.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

Wheat (*Triticum aestivum*) cv. Spark seeds were surface-sterilised as described by Gao *et al*. (2016), and germinated in a growth room in small containers. After 7 days, seedlings were harvested, flash frozen in liquid nitrogen, and then stored at -80 ℃ ready for use.

MOLECULAR CLONING OF *TaASN1, TaASN2* AND *TaASN3*

RNA was extracted using the hot phenol method (Verwoerd *et al*., 1989), with some modification, as described previously (Postles et al., 2016). It was used as a template for first-strand cDNA synthesis using SuperScriptIII® first-strand synthesis supermix (Invitrogen, supplied by Thermo Fisher Scientific, Hemel Hempstead, UK). The full-length coding sequences of *TaASN1*, *TaASN2* and *TaASN3* were then amplified by polymerase chain reaction (PCR). 'Forward' and 'reverse' primers for *TaASN1* were 5'-ccggaattcATGTGCGGCATACTGGC and 5'-ccgctcgagAACTCTCAATTGCGACACCAG (lower case letters denote additional nucleotides that were added to incorporate *Eco*RI and *Xho*I restriction sites at either end of the PCR product). 'Forward' and 'reverse' primers for *TaASN2* were 5'-ccggaattcATGTGCGGCATACTAGCGGTG and 5'-ccgctcgagAAGTCTCAATGGCAAC, while for *TaASN3* they were 5'-ccggaattcATGTGCGGCATCCTCGC and 5'-ataagaatgcggccgcAAACAGCAGCTGCTGGAACA. The additional nucleotides on the 'reverse' primer for *TaASN3* incorporated a *Not*I restriction site.

All products were amplified using Phusion® High-Fidelity DNA polymerase (New England Biolabs, Hitchin, UK). The cycling conditions were: 30 s denaturation at 98 ℃, followed by 35

cycles of 10 s at 98 ℃, 30 s at 63 ℃ and 30 s at 72 ℃, with a final extension period at 72 ℃ for 10 min. The resulting PCR products were purified using the Wizard PCR Clean-up system (Promega, Southampton, UK) and ligated into the pGEM-T Easy Vector (Promega, Southampton, UK) using the restriction sites incorporated during the PCR. Nucleotide sequence analysis was performed by MWG Biotech (Wolverhampton, UK) and contigs were assembled using ContigExpress or Geneious Version 8 (http://www.geneious.com; Kearse *et al*., 2012). Amino acid sequence alignments were also performed using Geneious Version 8.

GENOMIC ANALYSIS

DeCypher Tera-BLASTN Search Nucleic Query *vs.* Nucleic Database was used to assess the wheat asparagine synthetase gene sequences. The NR_Gene_v0.4 scaffold wheat genome was used as the database of choice (PLANT_T.aestivum_NRgene_v0.4_scaf, http://decypher1.rothamsted.ac.uk/decypher/cgi-bin/docfilter?file=/decypher/userindex.html), and the cDNA nucleotide sequences for *TaASN1* (GenBank BT009245), *TaASN2* (GenBank BT009049), and *TaASN3* (GenBank AK333183) were used as the query sequences.

The returned scaffolds were downloaded and aligned to the cDNAs using the Geneious Version 8 software package (pairwise alignment was run using the Geneious Alignment algorithm on its default settings; multiple alignments were run using the Consensus Align algorithm, again on its default settings). The aligned consensus sequences were then used to search the *Triticum aestivum* TGACv1 (Genomic sequence) database (http://plants.ensembl.org/Triticum_aestivum/Tools/Blast?db=core;tl=Igru1o47ao3Q1dXo-13756266) to assess chromosomal positioning. The returned genes from the TGAC database were then re-aligned to the original cDNA sequences to confirm gene identity.

TaASN4 was identified through its divergence from the other wheat asparagine synthetase sequences. The TGAC sequence was confirmed through re-alignments to both the TGAC and NR-Gene databases. BLAST searches using the PLANT_T.aestivum_nt_w7984 database were used to further confirm gene identity.

HETEROLOGOUS EXPRESSION OF *TaASN1*, *TaASN2* AND *TaASN3* IN *ESCHERICHIA COLI* (*E. COLI*)

The PCR products were excised from the PGEM®-T vector and ligated into the specialist expression vector, pET-30a (Novagen, UK) to produce plasmids pET-30a-TaASN1, pET-30a-TaASN2 and pET-30a-TaASN3. These were maintained in *E. coli* NovaBlue cells (Novagen, UK), which carry *recA* and *endA* mutations, and transferred to RosettaBlue™ cells (Novagen, UK) for high levels of expression of the ASN1-3 proteins. Single colonies of the cells carrying the plasmids were inoculated into medium containing 15 µg/mL kanamycin and 34 µg/mL chloramphenicol. The bacteria were grown at 37 ℃ with shaking until they had reached mid-log phase (OD 600 between 0.6 and 1.0). The culture was then split between two flasks, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to one of the flasks to a final concentration of 1 mM in order to induce expression of the asparagine synthetase gene carried by the plasmid. The other flask acted as an 'un-induced' control. The bacteria were incubated with shaking at 27 ℃ for a further 3 h, then harvested by centrifugation and stored at -80 ℃ until further use.

The use of the pET30a plasmid meant that the asparagine synthetase proteins were synthesized with a six-residue histidine N-terminal tag, and could therefore be extracted and purified using the nickel-nitrilotriacetic acid (Ni-NTA) purification system (Invitrogen, supplied by Thermo Fisher Scientific, Hemel Hempstead, UK). Bacterial cells were pelleted and lysed. Proteins in inclusion bodies were solubilized using NuPAGE® LDS-sample buffer and NuPAGE® Sample Reducing Agent and the proteins were separated on 4-12 % Bis-Tris gels (Invitrogen, supplied by Thermo Fisher Scientific, Hemel Hempstead, UK). Protein concentration was assayed using a Bicinchoninic Acid Kit (Sigma-Aldrich, Gillingham, UK).

WESTERN ANALYSIS

Monoclonal antibodies were produced by Abmart (Shanghai, China). Peptides were synthesized corresponding to probable epitopes in the C-terminal region of the TaASN1, TaASN2 and TaASN3 proteins where the amino acid sequences show less similarity with each other. Antibodies were raised to four different peptides for each of the three proteins. For western analysis, soluble proteins were separated by electrophoresis on NuPAGE® Novex® 4-12 % Bis-Tris gels and transferred to polyvinylidene fluoride (PVDF) membranes (13 cm × 8 cm) using the iBlot® Gel Transfer Device (Invitrogen, supplied by Thermo Fisher Scientific, Hemel Hempstead, UK). Immunodetection was performed with the antibody in a 1:1000 dilution for 2 h at room temperature, after which the membrane was incubated for 1 h at room temperature with 1:15000 horseradish peroxidase-conjugated goat anti-mouse IgG (Invitrogen, supplied by Thermo Fisher Scientific, Hemel Hempstead, UK). Bands representing proteins that had reacted with the anti-asparagine synthetase antibody were visualised with ECL™ Western Blotting Detection Reagents (GE Healthcare, Amersham, UK), and signals were quantified by scanning densitometry using Quantity One software (Bio-Rad Laboratories, Hemel Hempstead, UK).

ASPARAGINE SYNTHETASE ACTIVITY ASSAY

Purified asparagine synthetase proteins, TaASN1 and TaASN2, were added to an assay buffer of 100 mM HEPES (pH 7.6), 1.6 mM aspartate, 10 mM glutamine, 10 mM ATP, 10 mM $MgCl_2$ and 1 mM DTT and incubated at 30 ℃. Aliquots (100 µL) were removed after 1.5 min, 2.5 min, 3.5 min, 5 min, 15 min, 25 min and 35 min, placed in a 96-well filter plate and mixed with 100 µL of 10 % trichloroacetic acid to stop the reaction (Todd *et al*., 2008; Duff *et al*., 2011; Kudiyarova *et al*., 2013). Two replicate assays were done.

The free asparagine and glutamate produced in the reaction was detected after derivatisation with o-phthalaldehyde reagent (Sigma-Aldrich, Gillingham, UK). The fluorescent derivative was measured by high performance liquid chromatography (HPLC) using a Waters Alliance 2795 HPLC system fitted with a Waters 474 Scanning Fluorescence Detector (Waters, Elstree, UK). A Symmetry C_{18}, 4.6 × 150 mm column (for particle size 3.5 µm to 5 µm) (Waters, Elstree, UK) was used, and the fluorescence detector was set with an excitation wavelength of 340 nm and emission wavelength of 455 nm. For calibration, standards were used to provide areas under the HPLC peaks corresponding to asparagine and glutamate concentrations of 0, 5, 10, 15 and 20 nmol. The areas were modelled on the concentrations using linear regression, so that, by inverting the resulting linear equation, estimated concentrations of the amino acids and the asparagine synthetase enzyme could be made given HPLC areas for the sample aliquots taken at the seven sampling time points. Standards were run separately for each experiment.

MODELLING THE ASPARAGINE SYNTHETASE REACTION

A model for the reactions catalysed by asparagine synthetases TaASN1 and TaASN2 was constructed using the SNOOPY® tool (http://www-dssz.informatik.tu-cottbus.de/DSSZ/Software/) (Heiner *et al*., 2012) for designing, animating and simulating Petri Nets. The model was then exported to Copasi 4.16 (Build 104) (Hoops et al., 2006). Data for the reaction parameters were taken from the Brenda enzyme database (http://www.brenda-enzymes.org/).

RESULTS

MOLECULAR CLONING AND IDENTIFICATION OF THREE ASPARAGINE SYNTHETASE-ENCODING cDNAS FROM WHEAT (*TRITICUM AESTIVUM*)

Gao *et al*. (2016) identified three distinct asparagine synthetase gene nucleotide sequences in the GenBank database: *TaASN1* (Wang *et al*., 2005; GenBank AY621539; BT009245), *TaASN2* (GenBank BT009049) and *TaASN3* (GenBank AK333183). *TaASN1* and *TaASN2* were already annotated as asparagine synthetases, but the *TaASN3* entry had not been up to that point. Gao *et al.* also identified a fourth gene, *TaASN4*, from a BLAST search of wheat genome data (www.cerealsdb.uk.net; Wilkinson *et al*., 2012). *TaASN4* is present in cultivated and wild rice (*Oryza sativa* and *Oryza brachyantha*), *B. distachyon*, *Ae. tauschii*, foxtail millet (*Setaria italica*) and maize (*Zea mays*), as well as wheat (Gao *et al*., 2016), but to date has not been cloned from or characterized in wheat.

Gao *et al*. (2016) described the differential expression of *TaASN1*, *TaASN2* and *TaASN3* in different wheat tissues and in response to nitrogen and sulphur feeding, with expression of *TaASN2* in the embryo and to a lesser extent the endosperm of the grain during mid-development being far higher than the expression of any of the genes in any other tissue, although *TaASN1* showed most response to nutrition. This suggests that *TaASN2* expression in the grain is the primary determinant of asparagine levels, either for protein synthesis or accumulation in the free form, rather than import of free asparagine from other tissues, at least under normal (nutrient-sufficient) conditions, making *TaASN2* a potential target for genetic interventions to reduce free asparagine accumulation in wheat grain. However, modelling of the processes controlling the accumulation of free asparagine in order to confirm this requires information on the kinetic parameters of asparagine synthetase enzymes to add to the data on gene expression, and this was the aim of the current study.

To that end, *TaASN1*, *TaASN2* and *TaASN3* PCR products were amplified from wheat cv. Spark using primers designed from published sequences (see Materials and Methods Section). *TaASN1* from this variety was found to encode a protein of 585 amino acid residues with a molecular weight of 65.49 kDa, while *TaASN2* encoded a slightly smaller protein of 581 residues, molecular weight 65.06 kDa, and *TaASN3* a slightly larger protein of 591 residues, molecular weight 66.24 kDa. The nucleotide sequences have been deposited in the GenBank database and been given accession numbers KY937995, KY937996 and KY937997. Comparisons of the derived amino acid sequences of the proteins with those encoded by the nucleotide sequences already in the database confirmed that the three PCR products were derived from *TaASN1*, *TaASN2* and *TaASN3* (Table 1).

The amino acid sequences of the three proteins are aligned in Figure 1. All three share conserved amino acid residues typical of asparagine synthetases (highlighted in red in Figure 1), including the essential residues of a *purF*-type glutamine-binding site, Cys^{2}, Asp^{34} and His^{104} (Mei and Zalkin, 1989), and other residues important for glutamine binding and positioning that have been identified from the *E. coli* AsnB enzyme (Arg^{50}, Leu^{51}, Ile^{53}, Asn^{75}, Gly^{76}, Glu^{77} and Asp^{98}) (Larsen *et al*.,1999). Residues Thr^{316}, Thr^{317}, Arg^{319} and Cys^{523} are involved in the binding of aspartate and ATP (Boehlein *et al*., 1994a; 1994b; 1997a; 1997b), while Leu^{231}, Val^{267}, Ser^{341} and Gly^{342} have been recognized as the anchoring points for the AMP moiety (Larsen *et al*.,1999). Interestingly, Val^{267} is replaced with a different hydrophobic residue, Ile, in TaASN3. Lastly, the Ser, Gly, Gly, Leu, Asp, Ser motif beginning at position 233 is conserved in all of the asparagine synthetases characterized to date and may be involved in pyrophosphate binding (Richards and Schuster, 1998).

GENE STRUCTURE AND LOCATION

BLAST searches were performed of the wheat (*Triticum aestivum*) scaffold genome in the non-redundant genome database (NR_Gene_v0.4) using the cDNA sequences for *TaASN1*, *TaASN2* and *TaASN3* (Gao *et al*., 2016). This search also identified scaffolds for *TaASN4*, which to date has not been cloned from bread wheat (Gao *et al*., 2016). The returned scaffolds were aligned to the cDNAs to identify exons and introns, and the results confirmed by searches of the *Triticum aestivum* TGACv1 genomic sequence. The consensus sequences derived from these alignments were then used to search the Ensembl wheat database to determine the chromosomal positioning of the genes. Note that both the NR and TGAC genome data are from variety Chinese Spring.

The chromosomal positions and scaffold references for the genes are given in Table 2. *TaASN1*, *TaASN2* and *TaASN4* were all found to be single copy genes, located on Chromosomes 5, 3 and 4, respectively, of each genome (A, B and D), except that *TaASN2* was not present in the B genome. Analysis of unpublished wheat genome data (Alison Huttly, Rothamsted Research, personal communication) suggests that not all wheat varieties lack a *TaASN2* gene on Chromosome 3B, but the relative prevalence of the presence or absence of a B genome *TaASN2* gene cannot yet be assessed. In the case of *TaASN3*, there were two copies on Chromosome 1 of each genome, and these were given the names *TaASN3.1* and *TaASN3.2*.

The structures of the genes are shown in Figure 2, illustrating the considerable divergence of intron/exon patterns between the different genes, but conservation of structure within each group of homeologues. The three *TaASN1* homeologues, on Chromosomes 5A, B and D, are the shortest at approximately 3 kb from the ATG translation start codon to the translation stop codon, including 12 exons. The two *TaASN2* homeologues are approximately 4 kb in length, with 11 exons, and the three *TaASN3.1* and *TaASN3.2* homeologues just over and just under 6 kb, respectively, making them the longest group. The two *TaASN3* genes share a similar intron/exon pattern, with 15 exons. The three *TaASN4* homeologues are just under 4 kb in length, with 12 exons, except that the *TaASN4* gene on Chromosome 4B lacks exon 8. Clearly, this deletion may affect the activity of the enzyme encoded by the gene.

HETEROLOGOUS EXPRESSION AND PURIFICATION OF TaASN1, TaASN2 and TaASN3

The TaASN1, TaASN2 and TaASN3 PCR products were sub-cloned into vector pET-30a and

the resulting plasmids transformed into *E. coli* Rosetta DE3 cells to enable expression of the asparagine synthetase proteins. Use of this system meant that the proteins were synthesized with a six-residue histidine 'tag', enabling them to be purified on a Ni-NTA agarose column. Expression of the proteins was induced by addition of IPTG to the cell culture medium.

The result of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the expressed TaASN1, TaASN2 and TaASN3 proteins in both crude *E. coli* lysates and after purification on the column is shown in Figure 3. The proteins were solubilized by addition of NuPAGE® LDS-sample buffer, which contains lithium dodecyl sulphate at a pH of 8.4, and NuPAGE® Sample Reducing Agent, which contains dithiothreitol. An un-induced control is also shown (Figure 3) for each protein. The result showed the TaASN proteins to be highly expressed and readily purified.

WESTERN ANALYSIS; SCREENING OF PANEL OF ANTIBODIES

A panel of monoclonal antibodies was raised to peptides (Table 3) corresponding to epitopes within the variable C-terminal regions of the proteins (Figure 1) with the aim of identifying antibodies that showed high specificity for the TaASN proteins and could distinguish between them. A western blot of the heterologously-expressed TaASN1 proteins reacted with antibodies raised to two of the peptides, SKKPRMIEVAAP and GGSNKPGVMNTV, is shown in Figure 4. These two antibodies showed the highest specificity for the TaASN proteins, with the least non-specific binding, although all of the antibodies reacted with the TaASN proteins (not shown). However, none of the antibodies distinguished between the three asparagine synthetases. This was surprising because of the divergence of the amino acid sequences in this region (Figure 1). The SKKPRMIEVAAP epitope is present in TaASN1, while the GGSNKPGVMNTV epitope is at almost the same position in TaASN2 (indicated with a blue box in Figure 1). In each case there are only four identical residues and two conservative substitutions between the two proteins, and the similarity with TaASN3 is even lower. Nevertheless, TaASN1, TaASN2 and TaASN3 could be distinguished on the basis of size, with TaASN2 (65.06 kDa) migrating the furthest in the SDS-PAGE, followed by TaASN1 (65.49 kDa) and TaASN3 (66.24 kDa) (Figure 4).

PRODUCTION OF ASPARAGINE AND GLUTAMATE IN REACTIONS CATALYSED BY ASPARAGINE SYNTHETASES, TaASN1 and TaASN2

The production of asparagine and glutamate from aspartate and glutamine by TaASN1 and TaASN2 was measured in standard assays adapted from those described by Todd *et al*. (2008), Duff *et al*. (2011) and Kudiyarova *et al*. (2013). The reactions were sampled at 0, 1.5, 2.5, 3.5, 5, 15, 25 and 35 minutes and the asparagine and glutamate produced in the reaction were detected after conversion to a fluorescent derivative using o-phthalaldehyde reagent, separation by HPLC and detection of the fluorescent derivative with a scanning fluorescence detector. All three enzymes produced asparagine and glutamate, confirming that all three were asparagine synthetases. The concentrations measured for the reactions are given in Supplementary File S1 for the two replicate assays done. Data from the calibration of the HPLC areas using standard concentrations of asparagine and glutamate are also given. The results up to the 15 minute time point, by which time the concentrations of both asparagine and glutamate had plateaued, are shown graphically in Figure 5.

It was clear that the reactions catalysed by both asparagine synthetases proceeded much more rapidly than had been reported for heterologously-expressed maize or soybean enzymes (Todd *et al*., 2008; Duff *et al*., 2014). The reaction buffer contained 1.6 mM aspartate and 10 mM glutamine, meaning that glutamine was present in relative excess compared with aspartate. In both cases, the concentration of glutamate increased at a faster rate than the concentration of asparagine (Figure 5). By the 5 minute timepoint in both reactions, the concentrations of both products were at or close to their maximum, indicating that the concentration of the reactants had become depleted. Notably, at this point, the concentration of glutamate was more than double that of asparagine, and the measured concentration of asparagine was actually slightly higher than would be expected given the starting concentration of aspartate.

MODELLING THE ASPARAGINE SYNTHETASE REACTION

The SNOOPY® tool was used to construct a continuous Petri net model which incorporates underlying ordinary differential equations (ODEs), representing the reaction catalysed by asparagine synthetases, TaASN1 and TaASN2. The model was based on the reaction stages proposed by Gaufichon *et al*. (2010), with some modifications, and the experimental data, assuming mass action kinetics. A schematic diagram of the model is given in Figure 6. It comprises metabolites ('places' in Petri Net terminology) indicated by circles, and reactions ('transitions') indicated by squares, connected by arrows ('edges'). The concentration of metabolites is represented abstractly by numbers on places. The model comprises one compartment (cell) with eleven molecules (species): adenosine monophosphate (AMP), asparagine (Asn), asparagine synthetase enzyme (for the purpose of the modelling annotated as ASNe), asparagine synthetase enzyme complexed with glutamine (ASNe-Gln), asparagine synthetase enzyme complexed with ammonia (ASNe-NH_3), aspartate (Asp), adenosine triphosphate (ATP), β-aspartyl-complex (βAsp-AMP-ASNe-NH_3), glutamine (Gln), glutamate (Glu) and magnesium ions (Mg^{2+}). The four elementary biochemical reactions involved in the formation of asparagine are represented by the following equations:

- Reaction r1: ASNe + Gln → ASNe-Gln

- Reaction r2: ASNe-Gln → Glu + ASNe-NH_3

- Reaction r3: ASNe-NH_3 + Asp + ATP + Mg^{2+} (+ H_2O) → βAsp-AMP-ASNe-NH_3 + Mg^{2+} (+ PPi)

- Reaction r4: βAsp-AMP-ASNe-NH_3 → Asn + ASNe + AMP

Note that H_2O and PPi (pyrophosphate, $P_2O_7^{4-}$) are in parentheses because they are ubiquitous and therefore were not included in the model. The behaviour of each reaction is dependent on the corresponding parameter values and the initial concentrations of the metabolites. The model additionally includes a dissociation step for the ASNe-NH_3 complex (reaction 'D' highlighted in red in Figure 6), because this better fits the observed experimental data: Reaction D: ASNe-NH_3 → ASNe

The following ordinary differential equations were generated by the SNOOPY® Petri Net software (up to some naming adaptations to comply with the software requirements) to describe the mass action reactions determining the behaviour of the eleven metabolites in the model:

$$d\,Gln/dt = -(k1*Gln*ASNe) \quad (1)$$

$$d\,ASNe\text{-}Gln/dt = (k1*Gln*ASNe) - (k2*ASNe\text{-}Gln) \quad (2)$$

$$d\,ASNe/dt = (k4*\beta Asp\text{-}AMP\text{-}ASNe\text{-}NH_3) + (kD*ASNe\text{-}NH_3) - (k1*Gln*ASNe) \quad (3)$$

$$d\,Glu/dt = (k2*ASNe\text{-}Gln) \quad (4)$$

$$d\,ASNe\text{-}NH_3/dt = (k2*ASNe\text{-}Gln) - (k3*ASNe\text{-}NH_3*Asp*Mg^{2+}*ATP) - (kD*ASNe\text{-}NH_3) \quad (5)$$

$$d\,Asp/dt = -(k3*ASNe\text{-}NH_3*Asp*Mg^{2+}*ATP) \quad (6)$$

$$d\,\beta Asp\text{-}AMP\text{-}ASNe\text{-}NH_3/dt = (k3*ASNe\text{-}NH_3*Asp*Mg^{2+}ATP) - (k4*\beta Asp\text{-}AMP\text{-}ASNe\text{-}NH_3) \quad (7)$$

$$d\,Asn/dt = (k4*\beta Asp\text{-}AMP\text{-}ASNe\text{-}NH_3) \quad (8)$$

$$d\,AMP/dt = (k4*\beta Asp\text{-}AMP\text{-}ASNe\text{-}NH_3) \quad (9)$$

$$d\,ATP/dt = 0 \quad (10)$$

$$d\,Mg^{2+}/dt = 0 \quad (11)$$

The model structure and corresponding induced behaviour indicates that: (a) asparagine synthesis is dependent on the aspartic acid (aspartate), glutamine and ATP concentration; (b) when aspartic acid is depleted but glutamine is still available, asparagine synthetase will continue to hydrolyse glutamine to glutamic acid, which is consistent with the observed experimental data (Figure 5); (c) if all substrates are available except for ATP (i.e. ATP would only be an input to the system, unlike in the current model), the limiting factor becomes ATP.

The parameters (Table 4) were determined using the parameter estimation function of Copasi (version 4.16) (Hoops et al. 2006) based on the Hooke and Jeeves (1961) method. The values for the TaASN1 enzyme were defined using the following concentrations (mg/mL): ASNe between 1e-06 and 1e+06 with start value = 0.1; Glu between 1e-06 and 1e+06 with start value = 0.0; Asn between 1e-06 and 1e+06 with start value = 0.0, based on the data for Glu, Asn and ASNe provided in Supplementary File S1. The values for TaASN2 were the same, except for the start value of ASNe which was set at 0.09. The parameter estimation tasks were run for both enzymes for 2100 seconds with 2000 steps, size 1.05, with resulting rate values in units of mg/mL/s. The corresponding plots showing the time-series simulation results from the parameter fitting against the experimental data are given in Figure 7 for TaASN1 and Figure 8 for TaASN2, using initial concentrations of TaASN1 = 2.03 nmol/mL and TaASN2 = 2.10 nmol/mL.

DISCUSSION

Wheat is now known to contain four classes of asparagine synthetase genes, *TaASN1-4* (Gao *et al*., 2016). Our study established that there are single copies of *TaASN1*, *TaASN2* and

TaASN4, and two of *TaASN3*, and identified their chromosomal locations. The relatively simple structure of the gene family means that genetic interventions to reduce free asparagine accumulation and thereby acrylamide-forming potential in wheat grain are more likely to be successful. The antibodies raised in the study would be useful tools in the analysis of plants in which asparagine synthetase gene expression has been modified. The antibodies did not distinguish between TaASN1, TaASN2 and TaASN3, but it was possible to separate the enzymes on SDS-PAGE due to their slightly different sizes.

The study also showed that wheat asparagine synthetase enzymes, TaASN1 and TaASN2, can be expressed in *E. Coli* and analysed biochemically. Wheat asparagine synthetase activity has been measured before (Kudiyarova *et al*., 2013) but this was in plant extracts, so the results were not directly comparable to those obtained here. However, Todd *et al*. (2008) and Duff *et al*. (2014) analysed heterologously-expressed enzymes, the former from maize and the latter from maize and soybean. The reactions were not modelled, because their overall activity was very low, Todd *et al*. (2008) reporting a specific activity for asparagine production of 1-2 nmoles per min per mg of protein. The reaction buffer used by Todd *et al*. (2008) contained 1.6 mM aspartate and 1 mM glutamine, while Duff *et al*. (2011) used a buffer containing 1.6 mM aspartate and 2 mM glutamine, and the reactions were sampled over a period of 60 to 90 minutes. In this study, a buffer was used containing 1.6 mM aspartate and 10 mM glutamine, as well as 10 mM ATP and 10 mM $MgCl_2$, meaning that glutamine was present in relative excess compared with aspartate. The reactions catalysed by the wheat asparagine synthetases proceeded much more rapidly than had been reported for the maize or soybean enzymes. By the 5 minute timepoint, the concentrations of glutamate and asparagine were at or close to their maximum, indicating that the concentration of the reactants had become depleted.

A continuous Petri net model based on mass-action kinetics was constructed using SNOOPY® software to describe the reaction catalysed by asparagine synthetase, and a set of differential equations was generated to describe each part of the reaction. It was notable from the experimental data that the concentration of glutamate increased at a faster rate than the concentration of asparagine. Indeed, the product concentrations for both enzymes plateaued with the concentration of glutamate more than double that of asparagine, although the ratio of glutamate to asparagine was higher for TaASN1 than TaASN2. This indicates that the early stages of the reaction (r1 and r2 in the model) can proceed faster than and independently of the later stages (r3 and r4), consistent with the hypothesis proposed by Gaufichon *et al.* (2010) that steps r1 to r4 occur sequentially rather than simultaneously. So, despite the overall equation of the reaction being Glutamine + Aspartate + ATP $\rightarrow$ Glutamate + Asparagine + AMP + PPi, glutamate synthesis can proceed independently of asparagine synthesis when aspartate is not available.

Modelling of the reactions catalysed by TaASN1 and TaASN2 showed the two enzymes to be biochemically very similar except for the rate parameter (kD) for the dissociation step (Table 4). The careful fitting resulted in parameter values k1 to k4 which were within expected biochemical ranges (Meister, 1974). The dissociation reaction, D, which we postulate in order to be able to fit the overall model to the data, is currently not described in the literature. However, the higher kD value for TaASN1 could explain the higher ratio of glutamate to asparagine produced in the TaASN1 reaction compared with the TaASN2 reaction.

Gene expression analyses have shown *TaASN1* and *TaASN2* to be the most highly expressed asparagine synthetase genes in wheat grain, with *TaASN2* expression rising to 10 times that of *TaASN1* by mid-development (Gao *et al*., 2016). Given this and the similarity in the

biochemical data obtained for the two asparagine synthetases in the present study, we conclude that TaASN2 is the major enzyme synthesising asparagine in wheat grain, and therefore an appropriate target for genetic interventions to reduce free asparagine accumulation.

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Conflict of interest statement

The project was supported by a number of companies and organisations from the wheat supply chain.

TABLE 1. Amino acid sequence identity between the asparagine synthetases encoded by the PCR products amplified from wheat cv. Spark and wheat asparagine synthetases from the GenBank database.

	AY621539 (TaASN1)	BT009049 (TaASN2)	AK333183 (TaASN3)
Spark TaASN1	99%	88%	77%
Spark TaASN2	88%	100%	78%
Spark TaASN3	80%	78%	97%

TABLE 2. Chromosomal position and scaffold references for wheat (*Tritium aestivum*) cv. Chinese Spring asparagine synthetase genes (*TaASN1-4*). (http://decypher1.rothamsted.ac.uk/decypher/cgi-bin/docfilter?file=/decypher/userindex.html and http://plants.ensembl.org/Triticum_aestivum/Tools/Blast?db=core;tl=Igru1o47ao3Q1dXo-13756266)

Gene	Chromosomal Position	NR_gene v0.4 scaffold reference	TGACv.1 scaffold reference
TaASN1	5AL	10829_chr5A	376022_5AL:32188-35544
	5BL	86991_chr5B	404794_5BL:130599-132056
	5DL	24580_chr5D	438333_5DL:8628-10259
TaASN2	3AS	147930_chr3A	210989_3AS:73,515-78,055
	3DS	57063_chr3D	271746_3DS:53902-54460
TaASN3	1AL	72517_chr1A	004377_1AL:6267-12623
	1BL	94459_chr1B	032370_1BL:29616-29990
	1DL	40616_chr1D	061978_1DL:28081-28357
	1AL	81741_chr1A	002273_1AL:9143-9387
	1BL	95194_chr1B	031075_1BL:62894-63123
	1DL	86160_chr1D	061247_1DL:39750-39978
TaASN4	4AS	103865_chr4B	641929_U:112608-114682
	4BL	60431_chr4A	308427_4AS:31581-32567
	4DL	71289_chr4D	342578_4DL:59,248-59,851

TABLE 3. Epitopes used for the production of monoclonal antibodies for asparagine synthetases TaASN1, TaASN2 and TaASN3 from wheat.

Protein	Epitope identified from GenBank entries	Corresponding sequence in TaASN1-3 from cv. Spark	Position in protein
TaASN1 (GenBank AY621539)	HLPATIMAGTSK	HLPATILTGTSK	558-569
	IMAGTSKKPRMI	ILTGTSKKPRMI	563-574
	SKKPRMIEVAAP	SKKPRMIEVAAP	568-579
	MIEVAAPGVAIES	MIEVAAPGVAIES	573-585
TaASN2 (GenBank BT009049)	TVAVGGSNKPGV	TVAVGGSNKPGV	558-569
	GGSNKPGVMNTV	GGSNKPGVMNTV	562-573
	KPGVMNTVVPGV	KPGVMNTVVPGV	566-577
	MNTVVPGVAIET	MNTVVPGVAIET	570-581
TaASN3 (GenBank AK333183)	KAPASADPVFRP	KAPASVDPVLENAFHP	558-573
	DPVFRPPAHGES	DPVLENAFHPPAHGES	564-579
	PAHGESILVETG	PAHGESTLVKSA	574-585
	ILVETGVPAAAV	TLVKSAVPAAAV	580-591

TABLE 4. Rate parameters (mg/mL/s). Note that k1 refers to reaction r1 in the Petri net model shown in Figure 6, and likewise the other parameters, while kD refers to the dissociation reaction D.

Rate parameter	TaASN1	TaAsn2
k1	0.016	0.02
k2	3	3
k3	0.043	0.043
k4	10	10
kD	700	400

FIGURE LEGENDS

FIGURE 1.
Amino acid sequence alignment of TaASN1, TaASN2 and TaASN3 proteins from wheat (*Triticum aestivum*) cv. Spark. Identical residues at the same position are highlighted in black, except for residues known to be critical for the function of the enzyme (see text), which are highlighted in red. Similar residues at the same position (conservative substitutions) are highlighted in grey. The region corresponding to peptides used to raise the two monoclonal antibodies that showed highest specificity for the asparagine synthetase proteins is indicated with a blue box.

FIGURE 2
Diagrammatic representation of the gene structures of *TaASN1*, *TaASN2*, *TaASN3.1*, *TaASN3.2* and *TaASN4*.

FIGURE 3
SDS polyacrylamide gel electrophoresis of extracts of *E. coli* cells expressing wheat asparagine synthetases: TaASN1, TaASN2 and TaASN3. Expression of the proteins was induced by addition of IPTG to the bacterial cell cultures. An uninduced control is included for each protein, and each protein is also shown after purification on a nickel-nitrilotriacetic acid (Ni-NTA) agarose column. The arrow indicates the position of the expressed proteins.

FIGURE 4
Western blot of heterologously-expressed TaASN1, TaASN2 and TaASN3 proteins reacted with monoclonal antibodies raised to peptides SKKPRMIEVAAP and GGSNKPGVMNTV, as indicated.

FIGURE 5
Plots (means with standard errors from two replicates) showing the synthesis of asparagine and glutamate, in reactions catalysed by TaASN1 (top) and TaASN2 (bottom).

FIGURE 6
Model representing the reaction catalysed by asparagine synthetase, comprising metabolites (circles), and reactions (squares). The concentration of metabolites is indicated abstractly by numbers in the circles. The model features eleven molecules: AMP, ATP, asparagine (Asn), glutamine (Gln), glutamate (Glu), aspartate (Asp), asparagine synthetase enzyme (ASNe), ASNe complexed with glutamine (ASNe-Gln), ASNe complexed with ammonia (ASNe-NH_3), β-aspartyl-complex (βAsp-AMP-ASNe-NH_3), and magnesium ions. Note that for clarity, water and pyrophosphate are not included. The model was generated assuming that the reactions follow mass action kinetics. The dissociation step for the ASNe-NH3 complex is highlighted in red.

FIGURE 7
Time-series plots for parameter estimation against experimental data for TaASN1.

FIGURE 8
Time-series plots for parameter estimation against experimental data for TaASN2.

FIGURE 1

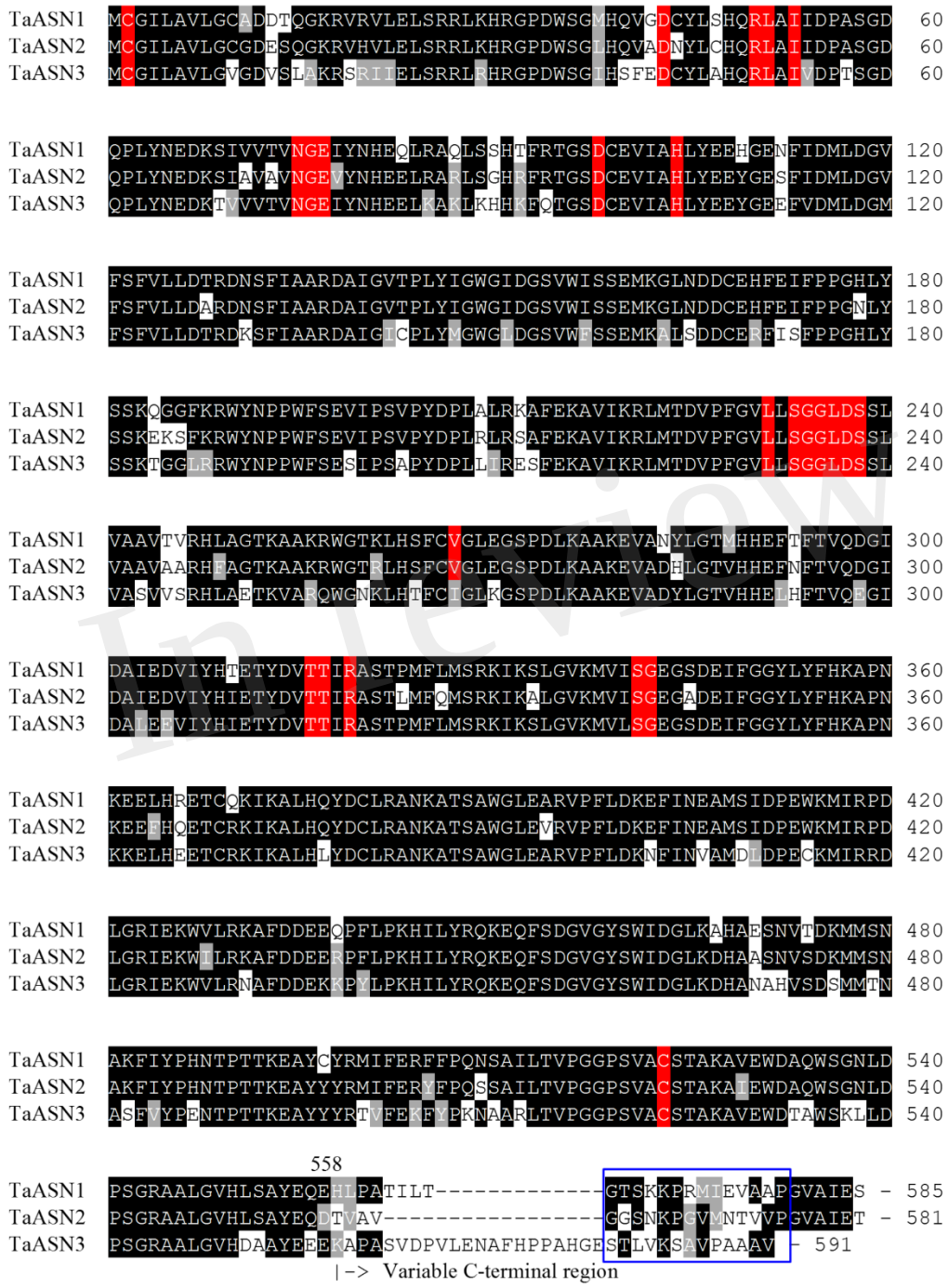

FIGURE 2

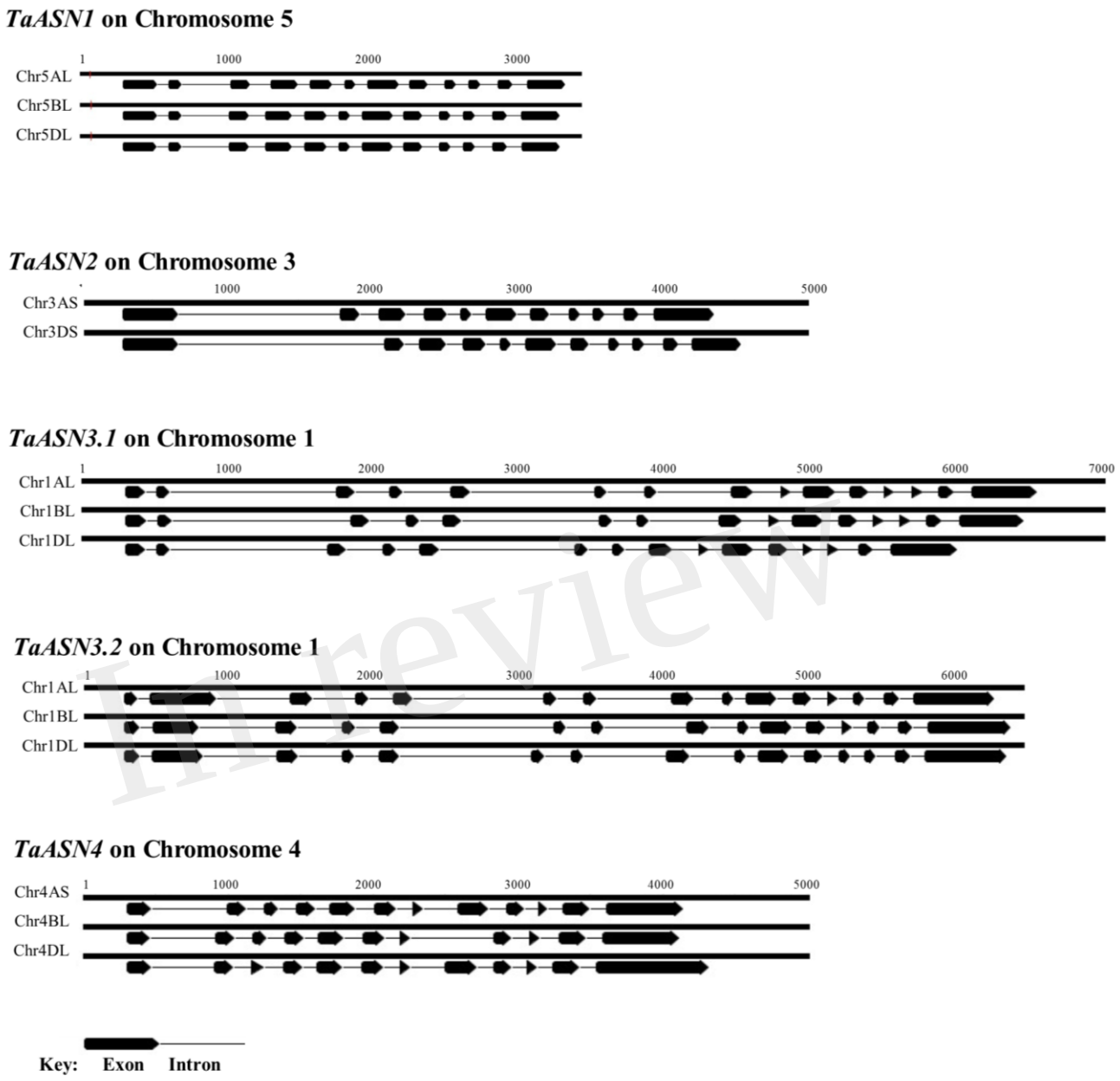

FIGURE 3

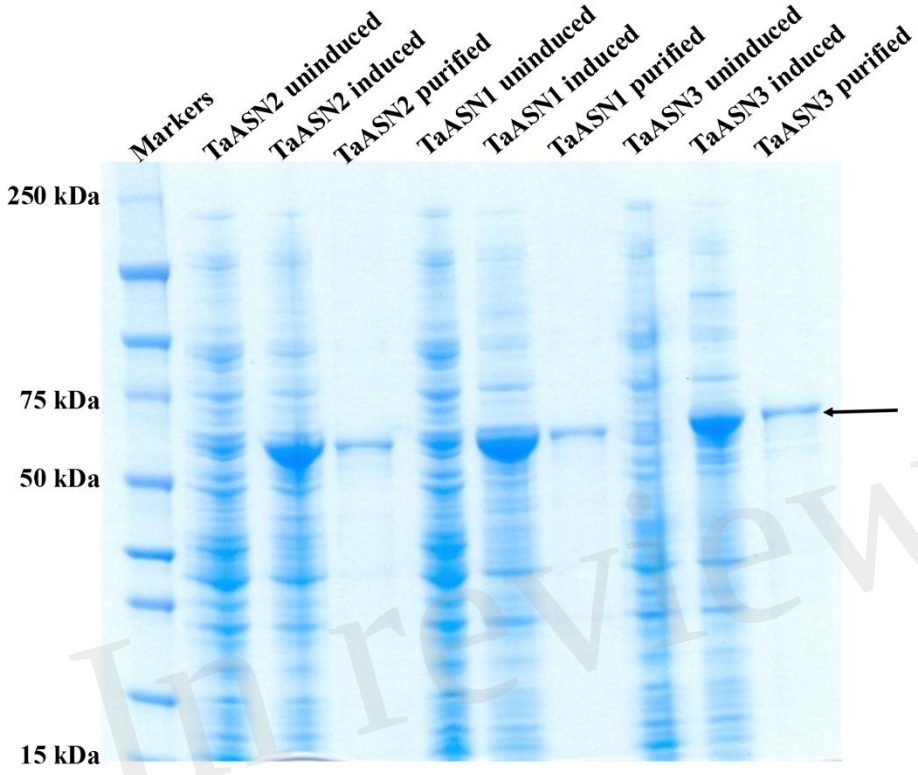

FIGURE 4

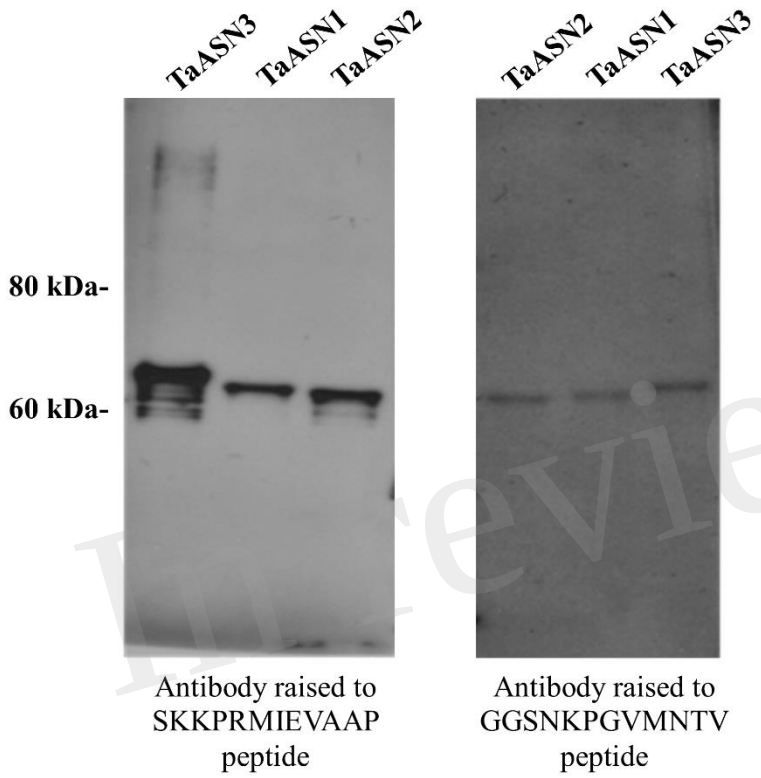

FIGURE 5

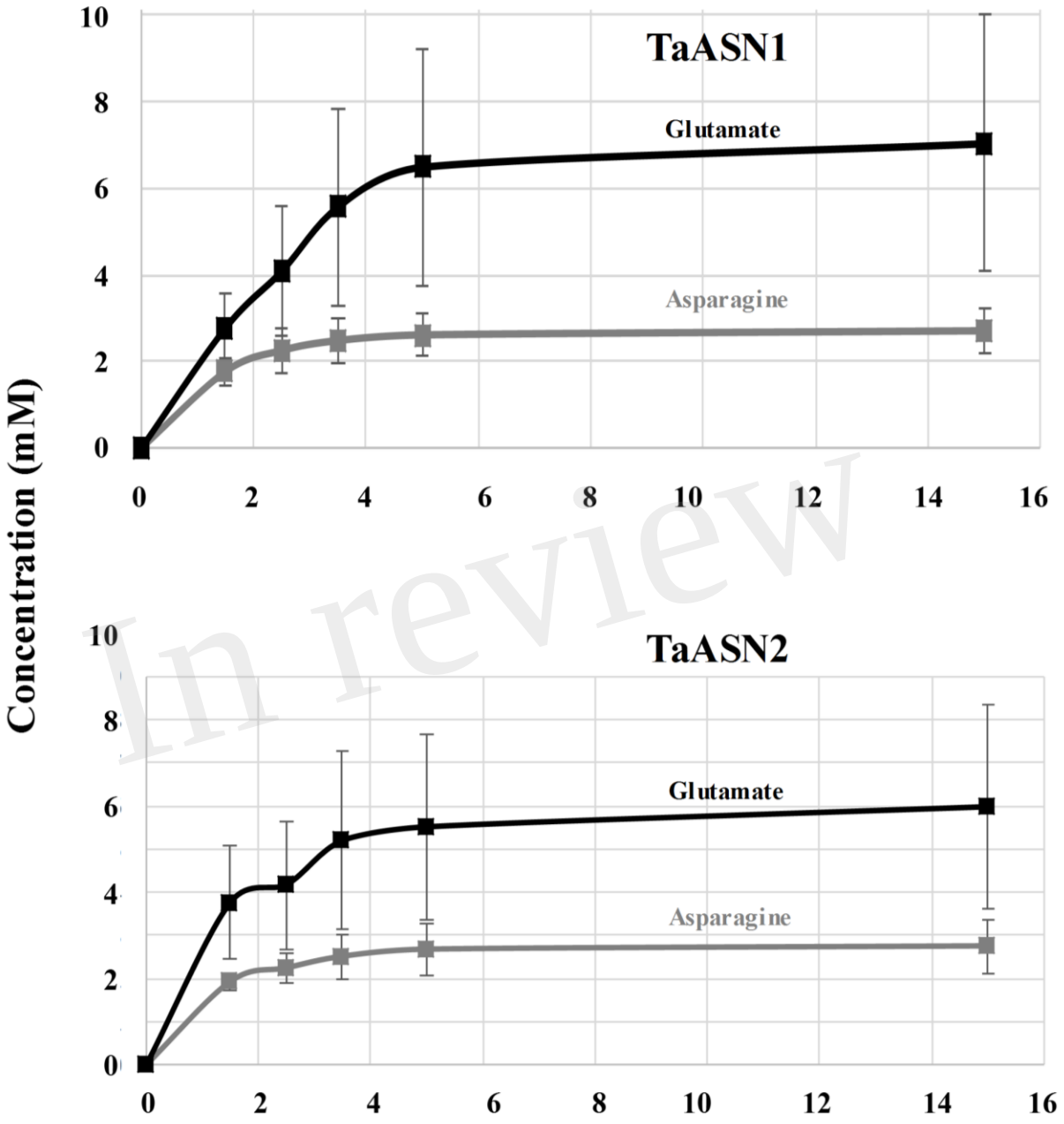

FIGURE 6

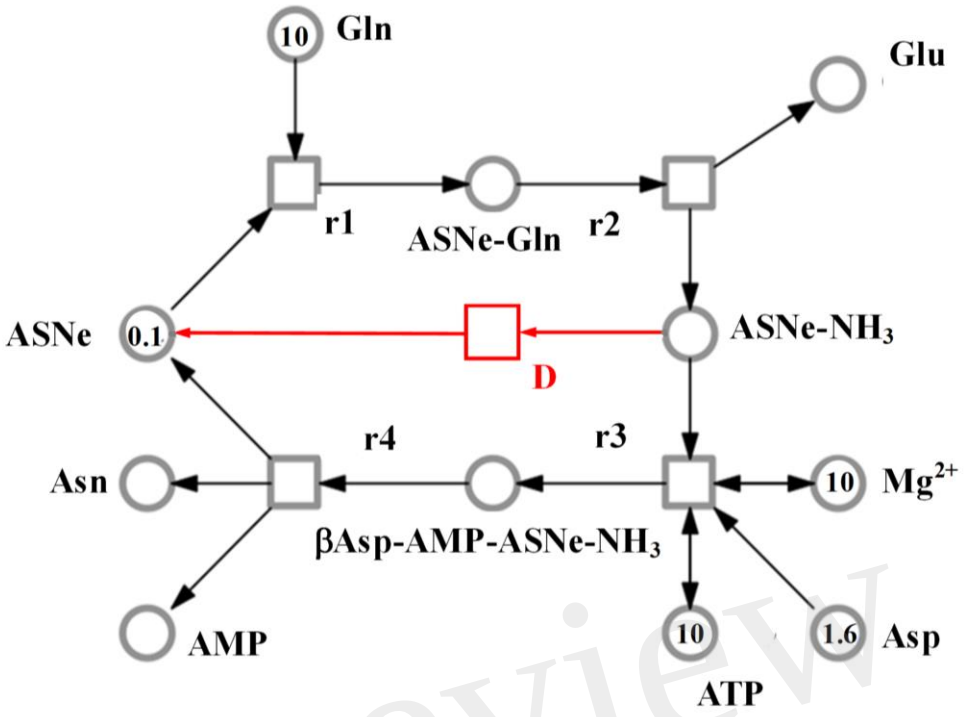

FIGURE 7

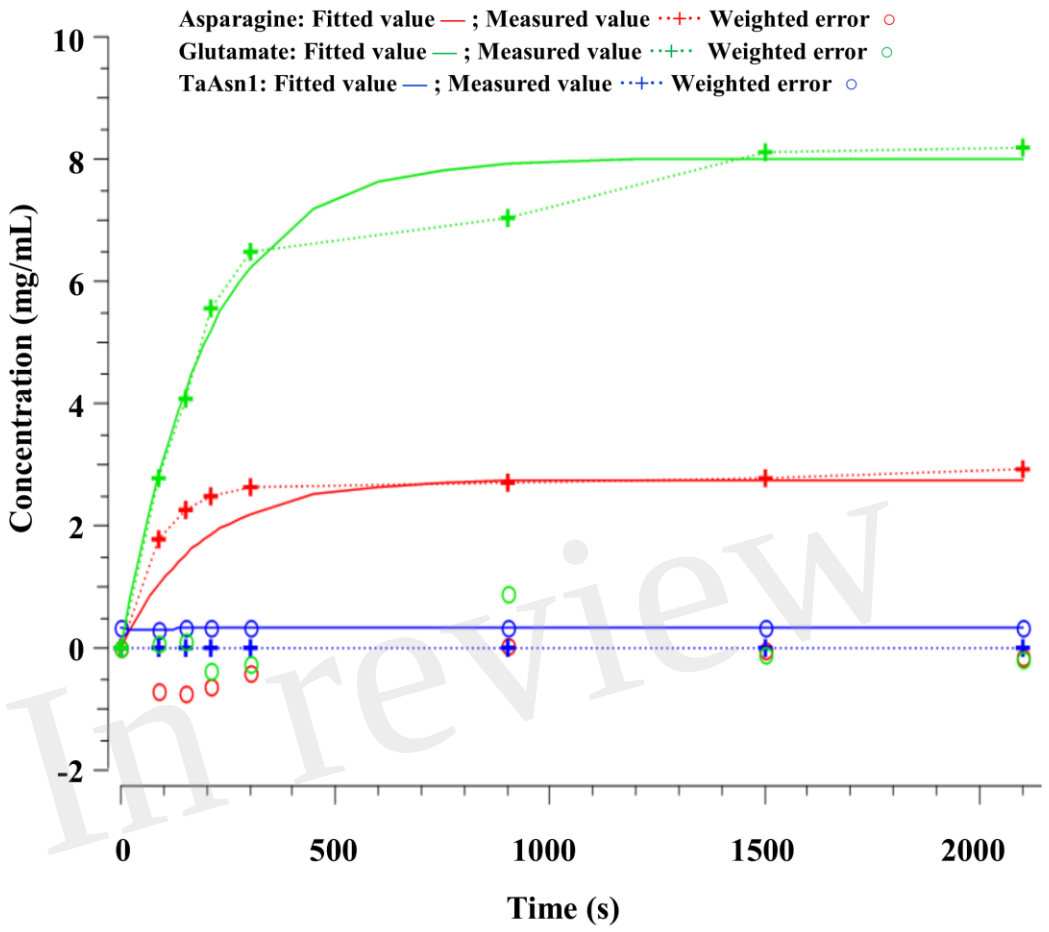

FIGURE 8

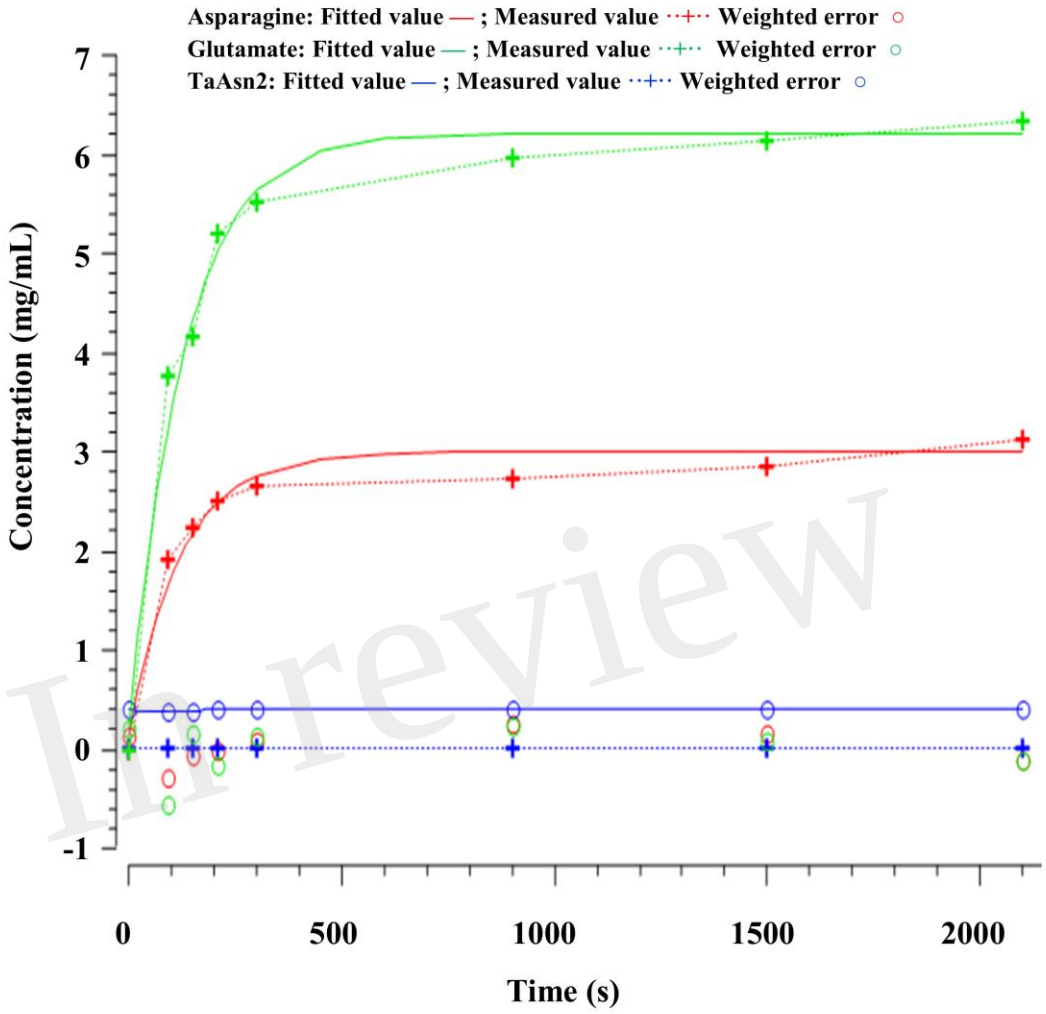

Figure 1.TIF

```
TaASN1   MCGILAVLGCADDTQGKRVRVLELSRRLKHRGPDWSGMHQVGDCYLSHQRLAIIDPASGD   60
TaASN2   MCGILAVLGCGDESQGKRVHVLELSRRLKHRGPDWSGLHQVADNYLCHQRLAIIDPASGD   60
TaASN3   MCGILAVLGVGDVSLAKRSRIIELSRRLRHRGPDWSGIHSFEDCYLAHQRLAIVDPTSGD   60

TaASN1   QPLYNEDKSIVVTVNGEIYNHEQLRAQLSSHTFRTGSDCEVIAHLYEEHGENFIDMLDGV  120
TaASN2   QPLYNEDKSIAVAVNGEVYNHEELRARLSGHRFRTGSDCEVIAHLYEEYGESFIDMLDGV  120
TaASN3   QPLYNEDKTVVVTVNGEIYNHEELKAKLKHHKFQTGSDCEVIAHLYEEYGEEFVDMLDGM  120

TaASN1   FSFVLLDTRDNSFIAARDAIGVTPLYIGWGIDGSVWISSEMKGLNDDCEHFEIFPPGHLY  180
TaASN2   FSFVLLDARDNSFIAARDAIGVTPLYIGWGIDGSVWISSEMKGLNDDCEHFEIFPPGNLY  180
TaASN3   FSFVLLDTRDKSFIAARDAIGICPLYMGWGLDGSVWFSSEMKALSDDCERFISFPPGHLY  180

TaASN1   SSKQGGFKRWYNPPWFSEVIPSVPYDPLALRKAFEKAVIKRLMTDVPFGVLLSGGLDSSL  240
TaASN2   SSKEKSFKRWYNPPWFSEVIPSVPYDPLRLRSAFEKAVIKRLMTDVPFGVLLSGGLDSSL  240
TaASN3   SSKTGGLRRWYNPPWFSESIPSAPYDPLLIRESFEKAVIKRLMTDVPFGVLLSGGLDSSL  240

TaASN1   VAAVTVRHLAGTKAAKRWGTKLHSFCVGLEGSPDLKAAKEVANYLGTMHHEFTFTVQDGI  300
TaASN2   VAAVAARHFAGTKAAKRWGTRLHSFCVGLEGSPDLKAAKEVADHLGTVHHEFNFTVQDGI  300
TaASN3   VASVVSRHLAETKVARQWGNKLHTFCIGLKGSPDLKAAKEVADYLGTVHHELHFTVQEGI  300

TaASN1   DAIEDVIYHTETYDVTTIRASTPMFLMSRKIKSLGVKMVISGEGSDEIFGGYLYFHKAPN  360
TaASN2   DAIEDVIYHIETYDVTTIRASTLMFQMSRKIKALGVKMVISGEGADEIFGGYLYFHKAPN  360
TaASN3   DALEEVIYHIETYDVTTIRASTPMFLMSRKIKSLGVKMVLSGEGSDEIFGGYLYFHKAPN  360

TaASN1   KEELHRETCQKIKALHQYDCLRANKATSAWGLEARVPFLDKEFINEAMSIDPEWKMIRPD  420
TaASN2   KEEFHQETCRKIKALHQYDCLRANKATSAWGLEVRVPFLDKEFINEAMSIDPEWKMIRPD  420
TaASN3   KKELHEETCRKIKALHLYDCLRANKATSAWGLEARVPFLDKNFINVAMDLDPECKMIRRD  420

TaASN1   LGRIEKWVLRKAFDDEEQPFLPKHILYRQKEQFSDGVGYSWIDGLKAHAESNVTDKMMSN  480
TaASN2   LGRIEKWILRKAFDDEERPFLPKHILYRQKEQFSDGVGYSWIDGLKDHAASNVSDKMMSN  480
TaASN3   LGRIEKWVLRNAFDDEKKPYLPKHILYRQKEQFSDGVGYSWIDGLKDHANAHVSDSMMTN  480

TaASN1   AKFIYPHNTPTTKEAYCYRMIFERFFPQNSAILTVPGGPSVACSTAKAVEWDAQWSGNLD  540
TaASN2   AKFIYPHNTPTTKEAYYYRMIFERYFPQSSAILTVPGGPSVACSTAKAIEWDAQWSGNLD  540
TaASN3   ASFVYPENTPTTKEAYYYRTVFEKFYPKNAARLTVPGGPSVACSTAKAVEWDTAWSKLLD  540

                      558
TaASN1   PSGRAALGVHLSAYEQEHLPATILT-------------GTSKKPRMIEVAAPGVAIES -  585
TaASN2   PSGRAALGVHLSAYEQDTVAV-----------------GGSNKPGVMNTVVPGVAIET -  581
TaASN3   PSGRAALGVHDAAYEEEKAPASVDPVLENAFHPPAHGESTLVKSAVPAAAV - 591
                                  |->  Variable C-terminal region
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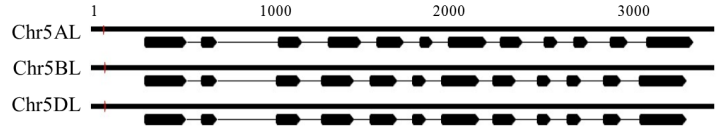
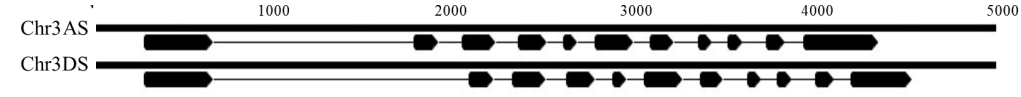
Figure 2.TIF

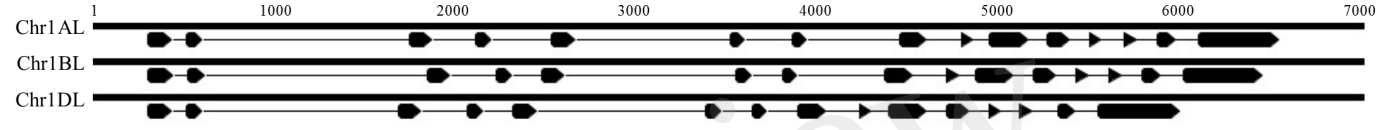
***TaASN1* on Chromosome 5**



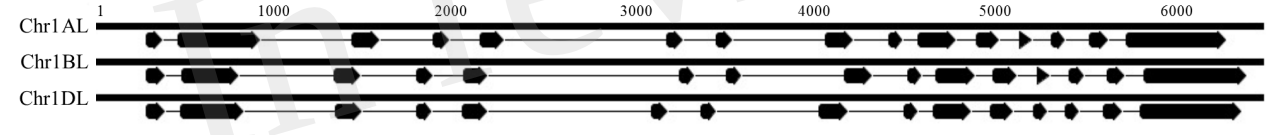
***TaASN2* on Chromosome 3**



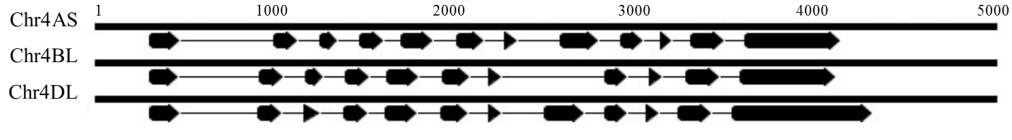
***TaASN3.1* on Chromosome 1**

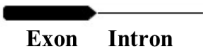


***TaASN3.2* on Chromosome 1**



***TaASN4* on Chromosome 4**





Key: Exon Intron

Figure 3.TIF

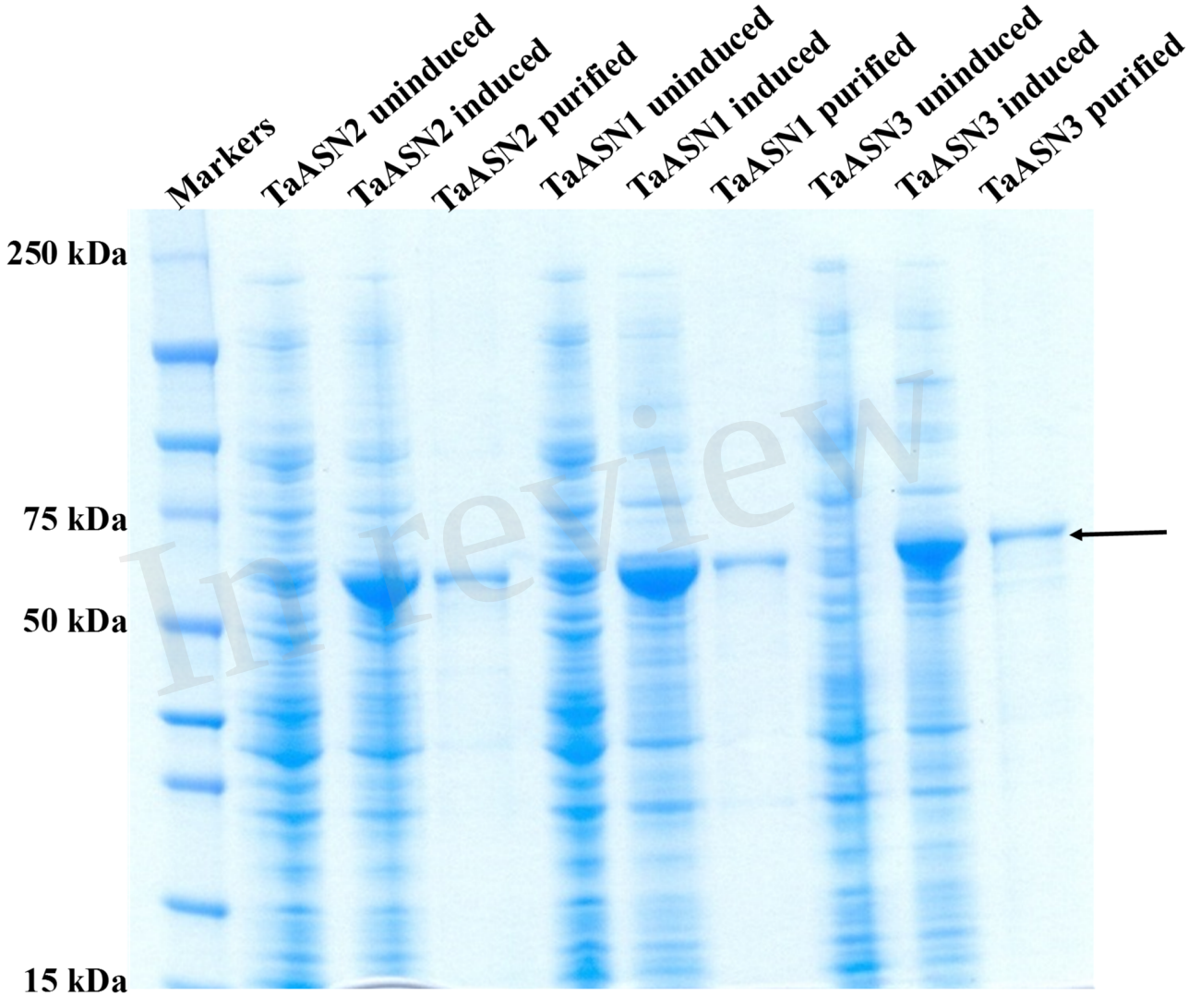
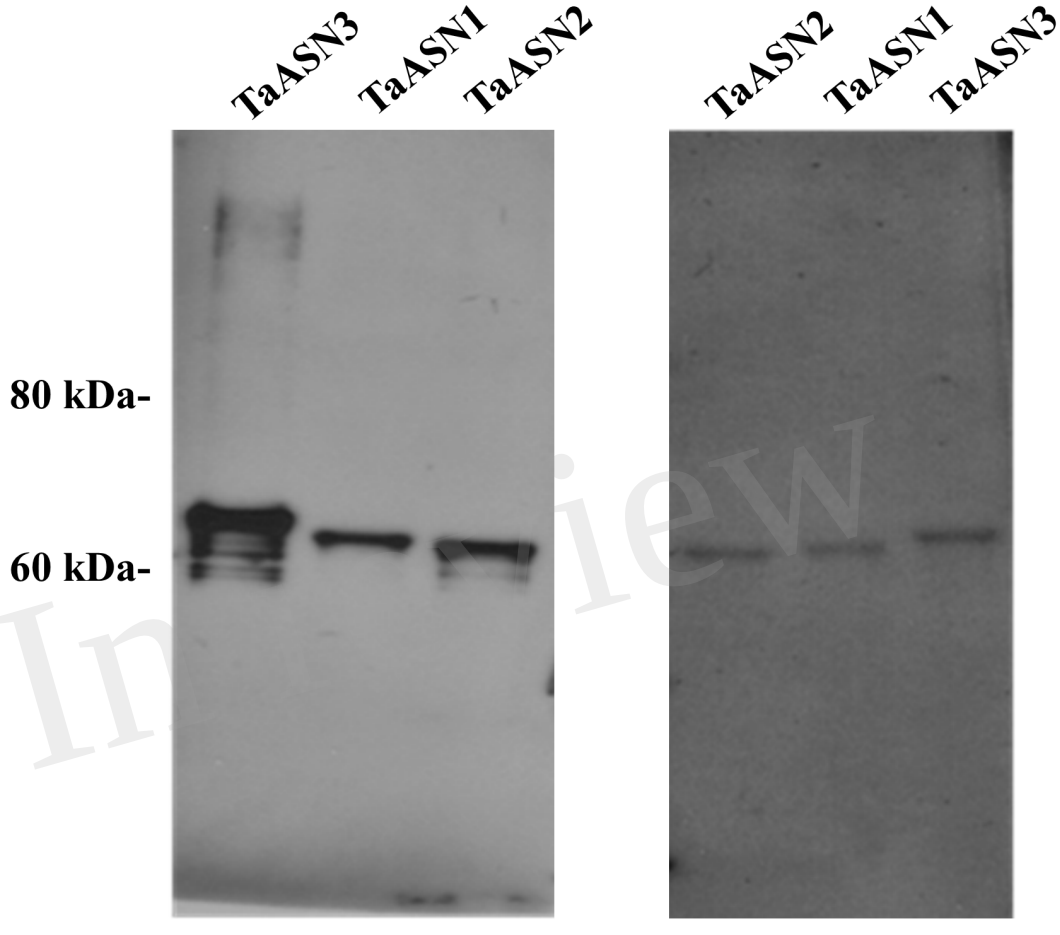


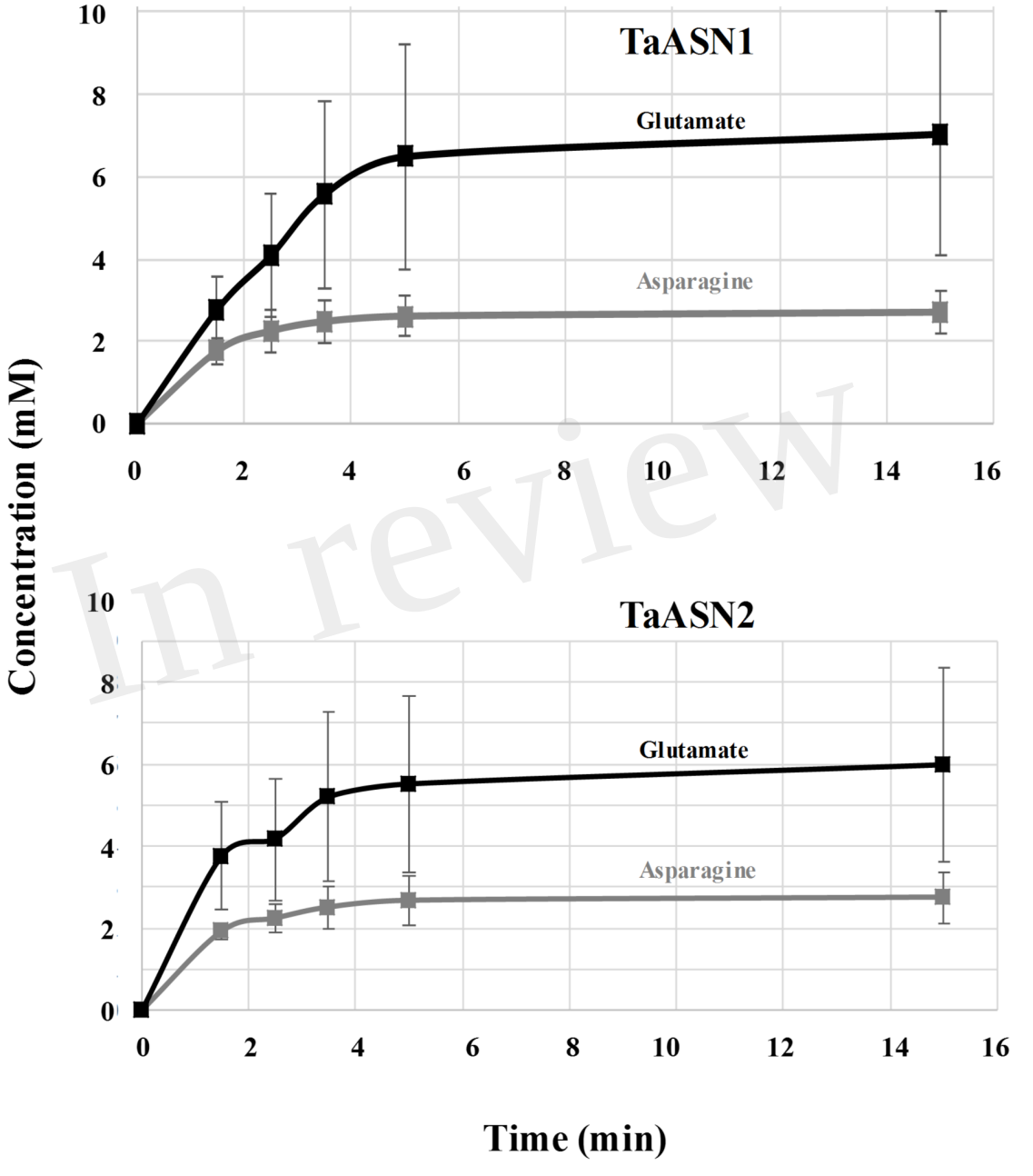
Figure 4.TIF



Antibody raised to SKKPRMIEVAAP peptide

Antibody raised to GGSNKPGVMNTV peptide

Figure 5.TIF



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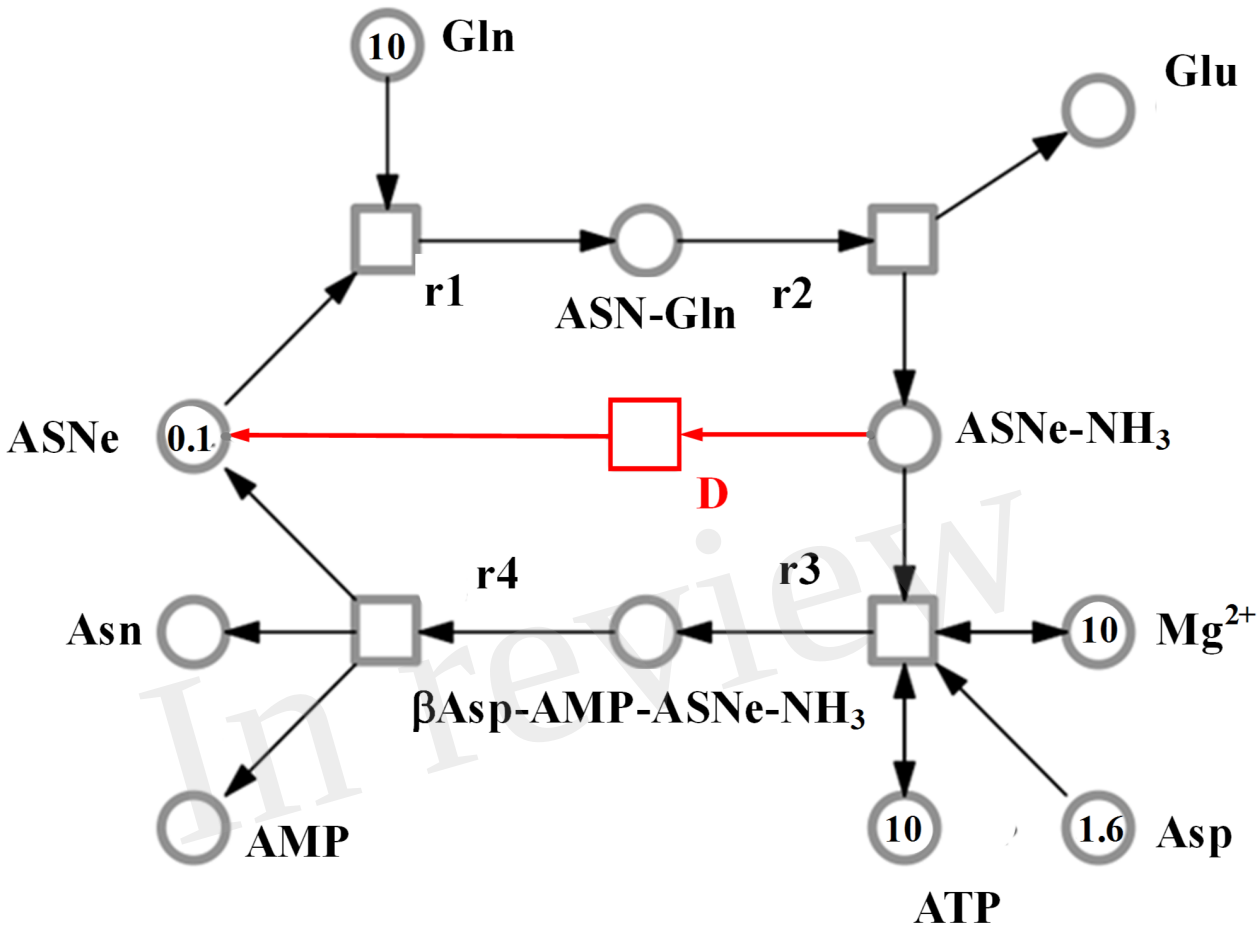

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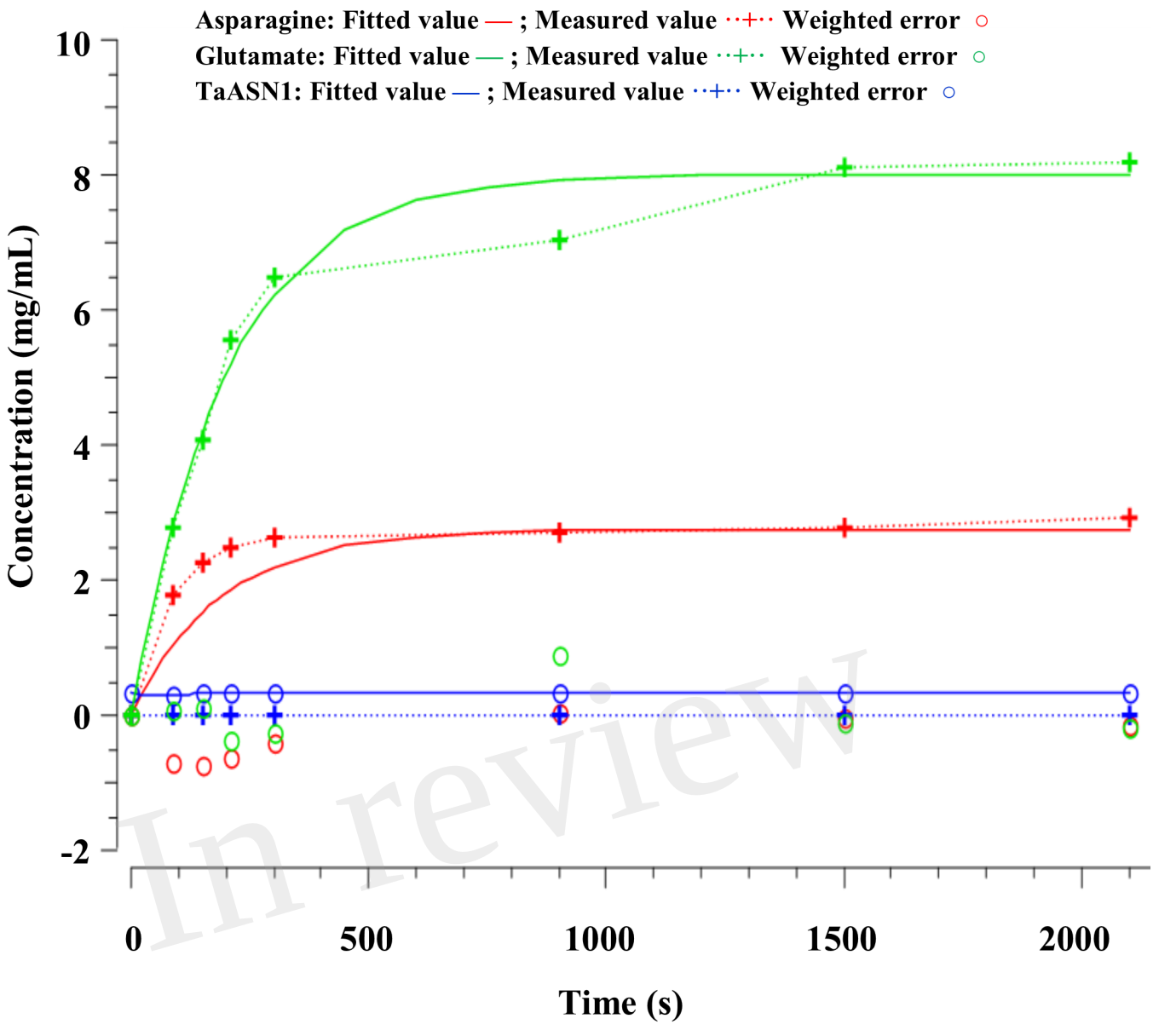

Figure 8.TIF