Molecular Immunology, Vol. 30, No. 18, pp. 1633–1638, 1993
Printed in Great Britain.

0161-5890/93 $6.00 + 0.00
Pergamon Press Ltd

IDENTIFICATION OF THE BINDING SITE OF TWO MONOCLONAL ANTIBODIES TO HUMAN PROTAMINE

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(*First received* 26 *July* 1993; *accepted in revised form* 27 *September* 1993)

Abstract—We have previously developed a number of monoclonal antibodies (Mabs) that bind to protamine. One of these antibodies, Hup1N, binds to human protamine 1 but not to protamine 2. In contrast, Mab HupA binds both protamine 1 and protamine 2. The epitopes for these two Mabs were observed to overlap, and were localized to the evolutionarily conservative amino-terminal region of protamine 1. This assignment is based on antibody binding to protamine from different species in which the protamine sequence is known, as well as analysis of antibody binding to synthetic peptides and synthetic peptides with specific amino acid substitutions.

INTRODUCTION

The protamines are a group of highly basic proteins that bind to DNA and replace the somatic histones and transition proteins during spermiogenesis in vertebrate sperm. A number of excellent reviews have been recently presented that deal with the biology of protamine at both the genetic and protein levels (Balhorn, 1989; Hecht, 1989*a*, *b*). Three different protamine molecules have been reported in human sperm and in rhesus monkey sperm (McKay *et al.*, 1986; Balhorn, 1989). These protamines are referred to a P1, P2a and P2b. Human P2a and P2b are related and are thought to be the products of a large precursor protein that is cleaved post-translation (Balhorn, 1989). Likewise, mouse P2 is synthesized as a precursor protein that is processed in late-step spermatids from 106 amino acids to the final 63 amino-acid sequence found in mature sperm (Balhorn, 1989; Yelick *et al.*, 1987). In contrast, no post-translational processing has been reported for P1. Sperm from other species such as mouse (Bellve *et al.*, 1981), hamster (Corzett *et al.*, 1987) and stallion (Pirhonen *et al.*, 1989, 1990) have been reported to contain P1 and a single form of P2. Finally, sperm from many species (e.g. bull, boar, ram) contain only a single protamine, P1 (Balhorn, 1989).

The complete amino acid sequence of P1 is known for many species including: bull (Coelingh *et al.*, 1972; Mazrimas *et al.*, 1986), boar (Tobita *et al.*, 1983), ram (Sautiere *et al.*, 1984), stallion (Belaiche *et al.*, 1987; Ammer and Henschen, 1987), mouse (from cDNA sequence) (Kleene *et al.*, 1985), rat (Ammer and Henschen, 1988), goat (Ammer and Henschen, 1988), rabbit (Ammer and Henschen, 1988), and human (McKay *et al.*, 1985, 1986; Ammer *et al.*, 1986; Gusse *et al.*, 1986). Balhorn (1989) has reported a partial amino acid sequence for Rhesus monkey P1. The amino acid sequence of P2 is known for a number of species including: mouse (Bellve *et al.*, 1988), human P2a and P2b (McKay *et al.*, 1985, 1986; Ammer *et al.*, 1986; Gusse *et al.*, 1986; Balhorn *et al.*, 1987), hamster P2 (a partial sequence) (Corzett *et al.*, 1987) and stallion P2 (Pirhonen *et al.*, 1989, 1990).

We have previously developed a number of monoclonal antibodies (Mabs) that bind to protamine (Stanker *et al.*, 1987, 1992). Some of these antibodies specifically bind human P1, some recognize P2 and others cross-react with both P1 and P2. Many of the P1-specific Mabs we isolated only recognize human P1. However, some of the Mabs cross-reacted with P1 from different species. The P2-specific Mabs cross-reacted with the P2 from most of the species known to contain a P2, and have been used in association with high-performance liquid chromatography to detect P2 precursors in rat sperm (Stanker *et al.*, 1992). The utility of these antibodies will be greatly enhanced if their epitopes can be accurately determined. Monoclonal antibody Hup1N was previously shown to bind P1 specifically, while Mab HupA cross-reacted with both P1 and P2 (Stanker *et al.*, 1987). In addition, both Mab Hup1N and HupA were previously shown to cross-react with protamine from a variety of species (Stanker *et al.*, 1987, 1992). In this paper we report on the identification of the epitope for Mabs Hup1N and HupA. Epitope assignment was accomplished by analysis of antibody binding to synthetic peptides corresponding to different segments of P1, and to synthetic peptides with substituted amino acids.

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MATERIALS AND METHODS

Sperm sources

Human semen was collected as part of a local program involving anonymous, healthy working donors 25–45 years of age. Bull, ram and boar semen were purchased from breeding services (Bull: American Breeders Service, DeForest, WI; Boar: Swine Genetics International, Cambridge, IA, and Ram: Dwane Garner, Dept. Animal Science, University of Nevada-Reno). Gerbils, *Meriones unguiculatus*, were obtained from Tumblebrook Farm Inc., West Brookfield, MA. Syrian hamsters, *Mesocricetus auratus*, were bred in our animal facility. The cauda epididymidis of adult Rhesus monkeys (California Regional Primate Center, University of California, Davis, CA) and male rats (retired *Rattus norvegicus* breeders purchased from Simonsen Labs, Gilroy, CA) were teased in 0.01 M Tris–HCl buffer pH 8.0, 0.15 M sodium chloride (Tris–HCl–saline) and the released sperm were filtered through a nylon mesh. The following protamine samples were isolated from sperm teased from epididymides provided by other investigators: chinchilla, *Chinchilla laniger*, and vole, *Microtus pennsylvanicus* (Larry Johnson, U.S. Department of Agriculture, Beltsvill, MD), cotton rat, *Sigmodon hispidus* (Frank Knight, Indiana University, Bloomington, IN), beagle, *Canis familiaris* (local veterinarian), mule deer, *Odocoileus hemionus* (local hunter), and rabbit, *Sylvilagus audubonii* (local hunter).

Peptides

The peptides used in these studies are depicted in Fig. 1 and are designated as follows: human 1 (Hu-1), amino acids 1–10 of human P1; Bull-1, amino acids 1–9; Bull-2, amino acids 10–17; Bull-3, amino acids 18–25; Bull-4, amino acids 26–33; Bull-5, amino acids 34–41; Bull-6, amino acids 42–50; Bull-7, amino acids 1–16 (note that a Gly has been substituted for Cys in position number 5). A glycine was substituted in the following peptides that correspond to amino acids 1–10 of bull P1: G-substituted peptide-1 (G for R at # 2); G-substituted peptide-2 (G for Y at # 3); G-substituted peptide-3 (G for R at # 4); G-substituted peptide-4 (G for C at # 5). All peptides used in this study except for Hu-1 were synthesized by the University of California, Protein Structure Laboratory (Davis, CA). The Hu-1 peptide was synthesized by Peninsula Laboratories (Burlingame, CA).

Protamine isolation and fractionation by HPLC

Whole protamine (i.e. the mixture of protamines 1 and 2) was isolated from sperm nuclei using a modification of the procedure for the mouse as described by Balhorn *et al.* (1977). The purified protamine from each species was lyophilized, weighed and adjusted to a stock concentration of 1 mg/ml. The isolated protamines were evaluated for purity by acid-urea gel electrophoresis (Panyim *et al.*, 1971).

Antibodies

Both monoclonal antibodies Hup1N and HupA have been previously described (Stanker *et al.*, 1987, 1992). Briefly, Mab Hup1N is a P1 specific antibody while HupA cross-reacts with P1 and P2. Both Mabs cross-react with protamine from a number of species. Mono-

Human P1	ARYRCCRSQSRSRYYRQRQRSRRRRRRSCQTRRRAMRCCRPRYRPRCRRH-coo⁻
Bull P1	ARYRCCLTHSGSRCRRRRRRRCRRRRRRFGRRRRRRVCCRRYTVIRCTRQ-coo⁻
Human 1 (1-10)	ARYRCCRSQS
Bull 1 (1-9)	ARYRCCLTH
Bull 2 (10-17)	SGSRCRRR
Bull 3 (18-25)	RRRRCRRR
Bull 4 (26-33)	RRRFGRRR
Bull 5 (34-41)	RRRVCCRR
Bull 6 (42-50)	YTVIRCTRQ
Bull 7 (1-16)	ARYRACLTHSGSRCRR
Peptide 1	AGYRCCLTHS
Peptide 2	ARGRCCLTHS
Peptide 3	ARYGCCLTHS
Peptide 4	ARYRGCLTHS

Fig. 1. Sequence of human and bull protamine 1 and of the synthetic peptides used in this study. The subscript for each peptide corresponds to the amino acid numbers of that peptide. The underlined amino acids in peptides 1–4 highlight amino acids that have been substituted in the bull 1–10 sequence.

clonal antibody Hup1M was produced as described by Stanker *et al.* (1992).

Enzyme-linked immunosorbent assays (ELISAs)

The direct binding enzyme-linked immunosorbent assays (ELISA) used to determine cross-reactivities were performed as described in Stanker *et al.* (1987). In all of the experiments, the 96-well microtiter wells were coated with the same amount of protamine (100 μl of a 1 μg/ml stock solution of protamine in distilled water was allowed to dry, at 37°C, onto the bottom of the microtiter wells). The percent cross-reactivity was calculated by comparing the area under the ELISA titration curves. Briefly, a perpendicular was dropped from the titration curve to the x-axis from the maximum and minimum antibody concentrations. The area in this region was determined and 100% assigned to the reaction with human protamine as antigen. All other protamine antigens were then expressed as a percent of the human value.

The ability of each Mab to bind the above peptides was evaluated using a competition ELISA (cELISA). The cELISA was performed as follows. Microtiter wells were coated with human protamine and subsequently blocked with a solution of 3% ovalbumin containing 1 μg/ml calf-thymus DNA as described above. The blocking step is necessary in order to mask reactive sites on the plastic microtiter plates not involved with antigen binding, and to neutralize any charges on the immobilized protamine molecules. Peptide competitors were then added to each well at the levels indicated in the figures, followed immediately by addition of the anti-protamine Mab. The antibody was added at a concentration that was determined in titration experiments to give approximately 50% of the maximum optical density and to be in the linear portion of the titration curve. Thus, for Hup1N this was a 1/10,000 dilution of the stock ascites fluid and for HupA a 1/50,000 dilution of stock ascites fluid. The plates were incubated for 1 hr at 37°C, and then they were processed as described above for the direct binding ELISA. The percent inhibition of control was calculated by comparing the O.D. (405 nm) observed in wells that did not have any peptide competitor added (control, or 100% activity) to the O.D. (405 nm) observed in wells in which peptide had been added. The results are expressed as percent inhibition of control (IC) and were calculated using the following formula:

$$\% \text{ UC} = [1-(\text{O.D.}_{405\text{nm}} \text{ test well}/\text{O.D.}_{405\text{nm}} \text{ control well})] \times 100.$$

The percent cross-reactivity of the antibodies to different peptide antigens was calculated by comparing the IC_{50} value obtained with the Hu-1 peptide with the IC_{50} values of the other peptides in competition ELISAs using the following formula:

$$\% \text{ cross-reactivity} = [1-(IC_{50} \text{ unknown}/IC_{50} \text{ human})] \times 100.$$

Table 1. Percent cross-reactivity of Hup1N and HupA to the protamines from the species listed

Species	% Cross-reactivity[a] Hup1N	Hup1M	HupA
Boar	92	0[b]	100
Bull	75	0	170
Chinchilla	139	0	100
Cotton rat	79	0	83
Cynomologus monkey	70	0	122
Dog (beagle)	105	0	100
Gerbil	63	0	125
Mouse (C57Bk)	71	0	63
Mule deer	73	0	75
Syrian hamster	82	0	66
Rabbit	0	0	0
Ram	77	0	100
Rattus norvegicus	90	0	140
Rhesus monkey	100	0	80
Vole	4	0	100

[a]In reference to the reactivity with human protamine which is set at 100%.
[b]0 = less than 0.5% cross-reactivity.

RESULTS

Cross-reactivity studies of Hup1N and HupA

Hup1N has previously been shown to have a high degree of cross-reactivity (70–100%) with PI from a number of species (Stanker *et al.*, 1992). A summary of this data is shown in Table 1 as well as data from identical studies with HupA and Hup1M. In these experiments antibody binding was evaluated using a direct binding ELISA in which protamine from different species were used as antigen. Typical ELISA titration curves for Hup1N and HupA on human and rabbit protamine are shown in Fig. 2. Hup1N reacted with all of the species tested except for rabbit and vole. Likewise, HupA showed significant levels of cross-reactivity with all species tested except for rabbit. In contrast, Hup1M did not cross-react with any of the species tested and appears to be absolutely specific for human PI.

The species listed in Table 1 for which amino acid sequence data have been published have a conserved

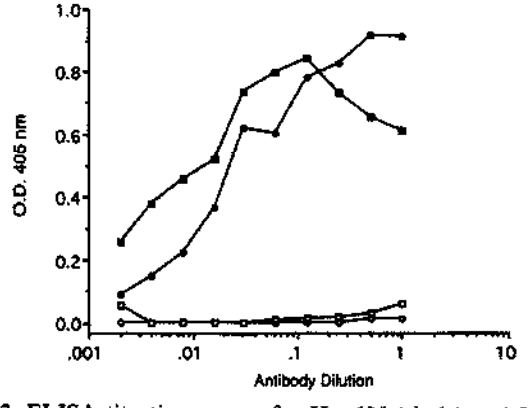

Fig. 2. ELISA titration curves for Hup1N (circle) and HupA (square) assayed on human protamine (solid symbols) versus rabbit protamine (unfilled symbols).

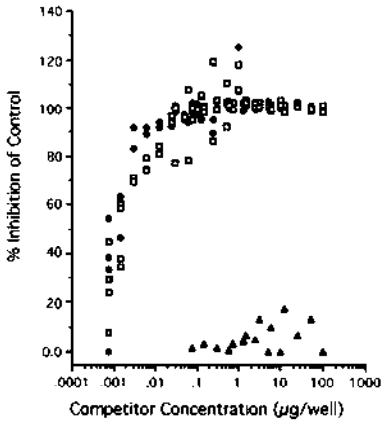

Fig. 3. Competition ELISA curves for Mabs Hup1N (circle), HupA (square) and Hup1M (triangle) using as competitor the Hu-1 peptide corresponding to amino acids 1–10 of human P1.

hexapeptide of amino acid sequence A-R-Y-R-C-C- corresponding to amino acids 1–6. An exception is rabbit, which has a Val in the number 1 position versus Ala. Since Hup1N also did not bind vole P1, an amino acid sequence was obtained for the amino end of vole P1. These studies resulted in the following partial sequence for vole P1 beginning at position number 1: A-R-Y-R-C-R. The inability of Hup1N to bind rabbit and vole protamine and the failure of HupA to bind rabbit P1, suggests that both Mabs are binding to the amino terminus of P1. In order to test this hypothesis, antibody binding was evaluated using a synthetic peptide, Hu-1, that corresponds to the first 10 amino acids of human P1. Direct binding ELISA experiments in which the Hu-1 peptide was conjugated to BSA and used as a coating antigen were performed (data not shown). Both Hup1N and HupA were observed to strongly bind the Hu-1 peptide. In contrast, Hup1M, also a P1 binding protein but specific for human P1, did not bind this peptide.

It is often difficult to obtain reproducible and quantitative binding using a direct binding ELISA. Thus, a competition ELISA (cELISA) format was adopted for the analysis of peptides. Figure 3 shows the competition ELISA data observed with Hup1N, Hup1M and HupA using the Hu-1 peptide as competitor. The inhibition of control value (IC_{50}) for both Hup1N and HupA is near 0.001 μg of peptide. As expected from the above direct binding ELISA studies, Hup1M did not bind the Hu-1 peptide, even at the highest level of peptide used (Fig. 3).

Since both Hup1N and HupA bound bull protamine 1 with near 100% cross-reactivity (Stanker *et al.*, 1987, 1992; and Table 1), a series of cELISAs using peptides that span the entire sequence of bull protamine (Fig. 1) were undertaken. The results from these experiments are summarized in Table 2. Bull peptide 1 was the only peptide of the set that resulted in inhibition of antibody binding. IC_{50} values of 0.0058 and 0.0025 μg/well were observed for Hup1N and HupA, respectively. The remaining bull peptides did not result in any significant inhibition even at the highest levels used (10 μg/well).

Since both Mabs strongly bound the bull-1 peptide relative to Hu-1, further cELISAs were performed to more critically map the epitope of these antibodies using a series of glycine substituted bull peptides (Fig. 1). The cross-reactivity data based on these cELISAs are summarized for both Hup1N and HupA in Table 2. Representative cELISA curves used to calculate the cross-reactivity for Hup1N are shown in Fig. 4. The percent cross-reactivity was calculated from these curves by comparing the IC_{50} values obtained with each of the four substituted peptides to the IC_{50} value obtained with nonsubstituted bull-1 peptide.

Table 2. Cross-reactivity[a] of Hup1N and HupA with the peptides indicated

Peptide	% Cross-reactivity[a] Hup1N	HupA
Bull-1	62	67
Bull-2	0[b]	0
Bull-3	0	0
Bull-4	0	0
Bull-5	0	0
Bull-6	0	0
Bull-7	0	13.5
Peptide-1	7.2	8.6
Peptide-2	70	0
Peptide-3	70	0
Peptide-4	5	58

[a]Cross-reactivity was calculated by comparison with the reactivity obtained with the Hu-1 peptide.
[b]0 = < 0.1% cross-reactivity.

CONCLUSIONS

The epitopes for the anti-protamine monoclonal antibodies Hup1N and HupA were identified by the studies presented here. Initial ELISA experiments using pro-

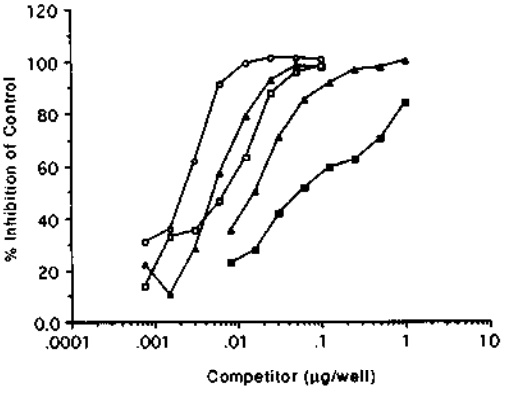

Fig. 4. cELISA curves obtained with Hup1N and various synthetic peptides having specific amino acid substitutions. See Fig. 1 for definition of peptides. Bull-1 peptide (open circle); G-substituted peptide 1 (filled triangle); G-substituted peptide 2 (unfilled triangle); G-substituted peptide 3 (unfilled square); G-substituted peptide 4 (filled square).

tamines from different species revealed that these antibodies cross-reacted with P1 from a large number of species representing different phylogenetic groups. These data suggested that the antibodies were binding to a highly conserved region of the protamine molecule. One such region was recently described by Balhorn *et al.* (1989), and it consists of the first six amino acids of the P1 molecule. This sequence A-R-Y-R-C-C-, is referred to as the conserved hexapeptide sequence and was found in all species tested except for rabbit and vole. Rabbit P1 is known to have a Val instead of an Arg at position number 1 (Ammer and Henschen, 1988). Thus, the observation that Hup1N and HupA failed to bind rabbit protamine was significant and suggested that the epitope for these antibodies was located near the amino terminal end of the P1 molecule. HupA bound vole protamine as well as human protamine. In contrast, Hup1N failed to bind vole protamine. Since the amino acid sequence of vole P1 was not available, we determined an amino acid sequence for the amino end of this P1. We found that vole P1, like rabbit P1 had a different hexapeptide sequence. Vole P1 was found to have an Arg at amino acid number 6 instead of the more commonly occurring Cys. Thus, the failure of Hup1N to bind vole protamine also suggested that, at least for Hup1N, the epitope was located in the amino portion of the P1 molecule. Furthermore it suggests that the Cys at amino acid position number 6 is involved with antibody binding. The observation that HupA bound vole protamine suggested that a Cys at this position was not critical for HupA binding. These results furthermore suggested that the epitopes for Hup1N and HupA were not identical. This latter suggestion was anticipated since Hup1N is specific for P1, while HupA has been shown previously to have a significant (i.e. 40%) cross-reactivity with P2 (Stanker *et al.*, 1987).

Next we evaluated the binding of these monoclonal antibodies to a synthetic peptide corresponding to the amino terminal end of human P1 (the Hu-1 peptide). Both a direct binding ELISA and a competition ELISA format were used. These experiments confirmed that the epitope for both Hup1N and HupA was indeed localized to the amino terminus of P1. The antibodies probably bind to the conserved hexapeptide region. In contrast, Mab Hup1M, an antibody that is absolutely specific for human P1 did not bind the Hu-1 peptide. Clearly, the epitope for Hup1M is not located at the amino end of the P1 molecule. Since Hup1N and HupA recognize bull protamine, a number of cELISAs were performed using a series of peptides (Fig. 1) that spanned the entire length of the bull P1 molecule. Only the bull-1 peptide, corresponding to amino acids 1–9 of bull P1, was recognized by Hup1N and HupA. While the direct binding ELISAs with intact P1 suggested a near 100% cross-reactivity, only 65% cross-reactivity was observed with the bull-1 peptide for both antibodies with the cELISA. However, both values represent a high level of binding, and suggest that positions 7, 8 and 9, which have different amino acids than found in human P1 (Leu for Arg at # 7, Thr for Ser at # 8, and His for Gln at # 9), are not major contributors to antibody binding. Clearly, these differences cannot be critical for antibody binding since only a 33–38% reduction in binding was observed. The peptide, bull-7 (Fig. 1) was also evaluated for binding to Hup1N and HupA. This peptide, in addition to being longer, also has an amino acid substitution at position number 5; an Ala is substituted for Cys. Hup1N failed to bind this peptide suggesting that a Cys at position number 5 is of critical importance for binding. HupA was observed to have a greatly reduced binding to the Bull-7 peptide suggesting that position 5 is also involved with the epitope for this antibody.

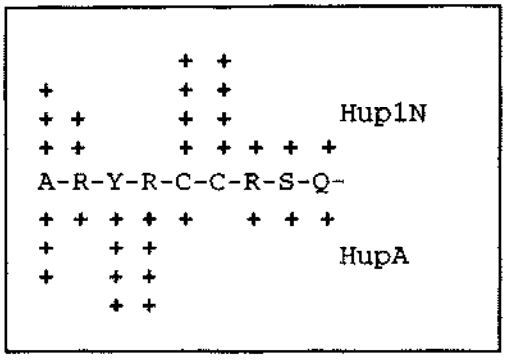

Fig. 5. A semiquantitative summary of the contribution of each amino acid to the binding of Hup1N (top) and HupA (bottom). The relative significance of each amino acid is proportional to the number of + symbols

Finally, a series of peptides corresponding to the bull-1 peptide but with single amino acid substitutions (Fig. 1) were evaluated using the cELISA. In each of these peptides a glycine was substituted for a different amino acid present in bull P1. Glycine was chosen because of its small size and its lack of charge. Hup1N and HupA were observed to differentially bind these peptides. For Hup1N binding was reduced by almost 100 fold by substitution of a Gly either at position 2 or position 5. Thus the amino acids at these positions appear to be critical for antibody binding. In contrast, only a 30% reduction in binding was observed when Gly was substituted at positions 3 and 4. Thus, while positions 3 and 4 are probably involved with binding, they appear to contribute less to the binding energy than positions 1, 2 and 5. HupA also differentially bound the glycine substituted peptides. The cELISA results for HupA binding suggest that the amino acids at positions 2, 3 and 4 each make a significant contribution to HupA binding, while the amino acid at position 5 contributes to a lesser extent.

The results of all of these studies suggest that the epitope for Hup1N and the epitope for HupA are localized to the amino terminal end of P1. Figure 5 is an attempt to visually display the relative importance of each amino acid for antibody binding and more clearly demonstrate the differences in epitope of these two antibodies. The greater the number of + symbols, the more critical is the amino acid. This representation clearly displays the overlapping nature of the epitopes for Hup1N and HupA, and is consistent with our observation on binding to intact protamines. Further

definition of the molecular nature of the epitopes would require analysis on a large number of peptides with multiple amino acid substitutions at each position.

Definition of the epitope at the level presented here however, greatly extends our understanding of these antibodies and strengthens their utility. Antibody binding to protamine-like molecules from species in which the presence of protamine has not yet been determined, especially the binding of Hup1N, can now be considered as strong evidence that the protein is a protamine 1 molecule. If nothing else, it could be concluded that binding suggests the presence of a protein with the conserved amino hexapeptide sequence. Antibody binding studies using sperm nuclear proteins from a large number of species are underway and will be the subject of further communications.

Acknowledgements—We thank Ms Michele Corzett and Mr Joe Mazrimas for their excellent technical assistance. This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract W-7405-ENG-48. Furthermore, mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

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