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6
7 **The impact of heating and cooling on the**
8 **physico-chemical properties of wheat gluten-water suspensions**

9
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22 *Abbreviations:* ACN, acetonitrile; db, dry basis; DTNB, 5.5'-dithio-bis(2-nitrobenzoic
23 acid); DTT, dithiothreitol; HMW-GS, high molecular weight glutenin subunits; HPLC,
24 high performance liquid chromatography; HT, holding time; LMW-GS, low molecular
25 weight glutenin subunits; P, Poise; RP, reversed phase; RVA, rapid visco analysis; SDS,
26 sodium dodecyl sulphate; SE, size exclusion; SH, sulphhydryl; TFA, trifluoroacetic acid

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29 *Keywords:* RVA, Wheat gluten, Heat treatment, Protein extractability, Cross linking

30

Abstract

The rapid visco analysis (RVA) system was used to measure rheological behaviour in 20% (w/v) gluten-in-water suspensions upon applying temperature profiles. The temperature profile included a linear temperature increase, a holding step, a cooling step with a linear temperature decrease to 50 °C, and a final holding step at 50 °C. Temperature and duration of the holding phase both affected RVA viscosity and protein extractability. Size-exclusion and reversed-phase HPLC showed that increasing the temperature (up to 95 °C) mainly decreased glutenin extractability. Holding at 95 °C resulted in polymerisation of both gliadin and glutenin. Above 80 °C, the RVA viscosity steadily increased with longer holding times while the gliadin and glutenin extractabilities decreased. Their reduced extractability in 60% ethanol showed that γ -gliadins were more affected after heating than α -gliadins and ω -gliadins. Enrichment of wheat gluten in either gliadin or glutenin showed that both gliadin and glutenin are necessary for the initial viscosity in the RVA profile. The formation of polymers through disulphide bonding caused a viscosity rise in the RVA profile. The amounts of free sulphydryl groups markedly decreased between 70 °C and 80 °C and when holding the temperature at 95 °C.

1. Introduction

Wheat gluten proteins consist of two major fractions: the monomeric gliadins which are readily soluble in aqueous alcohols and show viscous behaviour, and the polymeric glutenins which are elastic and insoluble in alcohol solutions (Veraverbeke and Delcour, 2002). Gliadins represent a heterogeneous mixture of proteins and α -, γ -, and ω -gliadins can be distinguished. Cysteine residues in α - and γ -type gliadins are all involved in intra-chain disulphide bonds. In contrast, ω -gliadins lack cysteine residues. Glutenin consists of

glutenin subunits (GS) of high molecular weight (HMW-GS) and low molecular weight (LMW-GS). The LMW-GS can be divided in B-, C-, and D-types. C-type LMW-GS resemble α - and γ -type gliadins much more closely than B-type LMW-GS. D-type LMW-GS can be classified with the ω -gliadins. They probably arose by a mutation in ω -gliadin genes resulting in the introduction of a cysteine residue. LMW-GS form both intra-chain and inter-chain disulphide bonds among themselves and with HMW-GS leading to glutenin polymers (Veraverbeke and Delcour, 2002).

Gluten proteins are susceptible to heat treatment. Heating wet gluten progressively decreases its breadmaking performance and at 75 °C most of its functionality is lost. The molecular size of the glutenin aggregates increases and, hence, their extractability decreases (Booth et al., 1980; Schofield et al., 1983; Weegels et al., 1994). At 100 °C, gliadins undergo similar changes. The extractability of gliadins from bread by 60% ethanol is much lower than that from flour, and α - and γ -gliadins are more affected than ω -gliadins (Wieser, 1998). The effects have been ascribed to sulphhydryl (SH) -disulphide interchange reactions induced by heat that affect all gluten proteins except the cysteine free ω -gliadins (Booth et al., 1980; Schofield et al., 1983). Morel et al. (2002) suggested that below 60 °C no change in free sulphhydryl groups occurs. Heating to at least 90 °C leads to disulphide bond linked aggregates and conformational changes affecting mostly gliadins and low molecular weight albumins and globulins (Guerrieri et al., 1996).

Although Kokini et al. (1994) proposed that crosslinks among gliadin molecules are formed above 70 °C in the absence of glutenin, others believe that gliadins cross-link only with glutenins (Redl et al., 1999; Singh and McRitchie, 2004). The incorporation of gliadin monomers in the glutenin network leads to a three-dimensional structure (Morel et al.,

2002). Due to crosslinking reactions, gluten viscosity levels off or increases upon heating (Attenburrow et al., 1990; Kokini et al., 1994). Not only increased temperature, but also mechanical shear upon mixing plays a role in the loss of sodium dodecyl sulphate (SDS) extractability of gluten proteins during analysis. Mixing favours protein reactivity, thereby lowering the energy of activation for protein solubility loss (Redl et al., 2003). Cooling favours the formation and retention of existing low energy interactions (Apichartsrangkoon, 1998; Hargreaves et al., 1995).

Heat treatment of wheat gluten protein and the resulting changes in rheological properties are of considerable importance for the characteristics of baked products and offer interesting features for non food applications. To increase our insight into the behaviour of gluten proteins during hydrothermal treatment, the Rapid Visco Analyser was used as a means to apply a temperature profile and simultaneously measure rheological changes. The extractability of the component gluten proteins during different temperature stages was analyzed with size-exclusion (SE)- and reversed-phase (RP)- high performance liquid chromatography (HPLC).

2. Experimental

2.1. Materials

Commercial wheat gluten [moisture content: 6.16%, crude protein content (N x 5.7): 78.9% on dry basis (db), starch content: 10.4% db] was from Amylum (Aalst, Belgium).

A gliadin and a glutenin enriched fraction were prepared from this commercial wheat gluten. Gluten (20.0 g) was extracted twice with 60% (v/v) ethanol (250 ml) (gliadin fraction) and once with deionised water (250 ml). After centrifugation (10 min, 10,000 g), the residue (glutenin enriched fraction) was freeze-dried and ground in a laboratory mill (IKA, Staufen, Germany). To remove ethanol the supernatant was dialysed (nine changes, 72 h) against 1 mM acetic acid, to conserve gluten functionality (Skerrit et al., 1996), and freeze-dried. Gliadin (crude protein content: 82.9% db), the glutenin enriched fraction (crude protein content: 67.9% db, gliadin content: 17.8% on protein basis) and respectively 1/4, 2/3 and 1/1 (w/w) mixtures of the two fractions were used for RVA analysis.

All reagents were of analytical grade.

2.2. Controlled heating and cooling

The Rapid Visco Analyser (RVA-4D, Newport Scientific, Sydney, Australia) was used to apply temperature profiles to 25.00 g of 20% (w/v) suspensions containing control gluten or gluten mixtures with different gliadin to glutenin ratios. Suspensions were hand-shaken and mixed (900 rpm for 20 s) at the start of the RVA analysis to obtain a homogeneous suspension. The temperature profile included a temperature increase from room temperature to 40 °C (in 1 min), a linear temperature increase to 95 °C, 90 °C or 80 °C at 3.95 °C/min, a holding step (5 to 60 min at 95 °C, 90 °C or 80 °C respectively), a cooling

step (7 min) with a linear temperature decrease to 50 °C, and a final holding step at 50 °C (13 min). The RVA system converts the current required to maintain constant mixing speed (160 rpm) of a paddle into a viscosity value in Poise (P; $0.1 \text{ kg m}^{-1} \text{ s}^{-1}$), the unit of dynamic viscosity. This viscosity value is further referred as RVA viscosity. The RVA was stopped at different points in the heating, holding and cooling phases of the profile and the gluten suspensions were frozen in liquid nitrogen, freeze-dried and ground in a laboratory mill (IKA, Staufen, Germany).

All RVA analyses were performed at least in triplicate. The standard deviations calculated from the initial viscosities, the minimal viscosities and the maximal viscosities were less than 6.5%.

2.3. Size-exclusion HPLC

SE-HPLC was conducted using a LC-2010 system (Shimadzu, Kyoto, Japan) with automatic injection. All samples (1.0 mg/ml) were extracted with a 0.05 M sodium phosphate buffer (pH 6.8) containing 2.0% sodium dodecyl sulphate (SDS) and loaded (60 µl) on a Biosep-SEC-S4000 column (Phenomenex, Torrance, United States). The elution solvent was (1:1, v/v) acetonitrile (ACN)/water containing 0.05% (v/v) trifluoroacetic acid (TFA). The flow rate was 1.0 ml/min at a temperature of 30 °C (Veraverbeke et al., 2000) and eluted protein was detected at 214 nm.

The elution profiles were divided into two fractions using the lowest absorbance reading between the two peaks as the cutoff point. The first fraction corresponds to the amount of SDS extractable glutenin, the second can be assigned to the amount of SDS extractable gliadin. Total SDS extractable protein, gliadin and glutenin were calculated from the peak

areas and expressed as percentage of the peak area of unheated gluten extracted with the SDS buffer in the presence of 1.0% dithiotreitol (DTT).

2.4. Reversed-phase HPLC

Samples (100.0 mg) were extracted three times with 3.0 ml 60% (v/v) ethanol (gliadin extract) and three times with 3.0 ml 0.05 M Tris/HCl buffer (pH 7.5) containing 50% propan-1-ol, 2.0 M urea and 1% (w/v) DTT and kept under nitrogen (reduced glutenin extract). The gliadin and glutenin extracts were loaded (80 µl) on a Nucleosil 300-5 C8 column (Machery-Nagel, Düren Germany). The elution system consisted of deionised water + 0.1% (v/v) TFA (A) and ACN + 0.1% TFA (v/v) (B). Proteins were eluted with a linear gradient from 24% B to 56% B in 50 min and detected at 214 nm.

α -Gliadin, γ -gliadin, ω -gliadin, B/C-LMW-GS, D-LMW-GS and HMW-GS were distinguished based on absorbance minima between specific peaks as outlined earlier by Wieser et al. (1998).

2.5. Protein content determination

Protein contents were determined using an adaptation of the AOAC Official Dumas Method to an automated Dumas protein analysis system (EAS variomax N/CN, Elt, Gouda, The Netherlands) (AOAC, 1995).

2.6. Free sulphydryl (SH) determination

Free SH groups were determined colorimetrically after reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). Samples (1.0-2.0 mg of protein/ml) were shaken for 60 min in

0.05 M sodium phosphate buffer (pH 6.5) containing 2.0% (v/v) SDS, 3.0 M urea and 1.0 mM tetrasodium ethylenediamine tetra acetate. DTNB reagent (0.1% w/v in sample buffer, 100 µl) was mixed with 1.0 ml sample and the extinction at 412 nm was determined 45 min after centrifugation (3 min, 11 000 g). Absorbance values were converted to amounts of free sulphhydryl using a calibration curve with reduced glutathione (Veraverbeke et al., 2000).

3. Results and Discussion

3.1. The effect of heating and cooling

Gluten suspensions showed a substantial RVA viscosity (1300-1500 cP) which decreased when the temperature was raised to 90 °C (Fig. 1). In the holding step (95 °C), the RVA viscosity steadily increased. During cooling, the RVA viscosity decreased again and, in the final holding step at 50 °C, no viscosity changes were observed. In this thermal process, the total amount of SDS extractable protein decreased to 40% in the final holding step. The heating step progressively reduced the amount of SDS extractable glutenin, while that of extractable gliadin remained constant. Holding at 95 °C decreased the amounts of both extractable glutenin and gliadin (Fig. 1). The decrease in RVA viscosity in the heating step was mainly due to the rise in temperature, because shearing at room temperature caused only a small decrease of RVA viscosity (results not shown). The decrease of RVA viscosity can be ascribed to changes in physico-chemical properties of the gluten proteins such as conformational changes (Guerrieri et al., 1996, Weegels et al., 1994) and a loss of hydrogen bonds which readily break on heating (Apichartsrangkoon, 1998). The decrease in extractability of gluten protein during the holding step and the increase of RVA viscosity

suggest formation of protein aggregates of increased molecular size impacting the rotation of the RVA paddle. The sudden decrease in apparent viscosity during cooling was due to the protein aggregates associating tightly and sticking to the paddle.

Fig. 2 shows the amounts of the different gliadin types and glutenin subunits during heating and cooling in the RVA. Between 70 and 95 °C, the extractabilities of α - and γ -gliadins decreased slightly, while that of ω -gliadins remained constant (Fig. 2a). The most drastic changes took place in the holding step at 95 °C with large reductions in α - and γ -gliadin extractabilities compared to their (maximal) extractability at 70 °C (40% and 48% respectively). During the holding step, the amount of ω -gliadins was reduced by 20%. In the cooling step, the amounts of extractable α - and γ -gliadin decreased further. At the end of the thermal process, the extractability of ω -gliadins (76%) was reduced less than that of α - (49%) and γ -gliadins (45%). The sharp decrease in extractable gliadin amounts during the holding step (Fig. 2a) was accompanied by a significant increase in the apparent amounts of the glutenin subunits (Fig. 2b), suggesting that a major portion of gliadins, unextractable in 60% ethanol after heat treatment, became extractable in the glutenin fraction. This resulted in an apparent increased proportion of B/C-LMW-GS (84% increase) after holding 5 min at 95 °C (Fig. 2b), but there was also an apparent increase in D-LMW-GS and HMW-GS fraction (23% and 26% respectively) (Fig. 2b). The sum of the gliadins and glutenins remained constant during heating, holding and cooling.

3.2. The effect of holding time and temperature

Heating and cooling gluten suspensions had a strong impact on RVA viscosity and protein extractability. Viscosity increased at 90 °C and during holding at 95 °C, while viscosity

decreased in the cooling phase. To further examine these observations, the time of holding and the holding temperature were varied and evaluated in terms of their impact on RVA viscosity and protein extractability.

On extending the holding phase the RVA viscosity reached a maximum after 35 min at 95 °C. Longer holding times at 95 °C resulted in a slow viscosity decrease (Fig. 3). Large protein aggregates were formed which initially increased the RVA viscosity. Due to the constant mixing the protein aggregates oriented themselves in the stirring direction. This shear thinning effect was reflected in a slow viscosity decrease after 35 min at 95 °C. This effect has also been described for starch-water suspensions where alignment of the soluble starch molecules during holding leads to a decrease in viscosity (Hoseney, 1994). Subsequently cooling caused a strong viscosity decrease. Cooling favoured association of the protein aggregates and, as indicated earlier, led them to stick to the paddle causing the abrupt viscosity decrease.

Holding at 95 °C for 60 min decreased the amount of SDS extractable protein (Table 1). The holding step had a strong impact on gliadin extractability. Holding gluten for 15 min at 95 °C reduced the SDS extractability of gliadin by more than 50% and a holding time of 40 min led to a reduction of gliadin extractability by 70% (Table 1). Most of the glutenin became unextractable during heating and the first 5 min of holding at 95 °C.

In the holding step at 95 °C, the amount of 60% ethanol extractable gliadin decreased (Fig. 4a). The amount of α -gliadin and γ -gliadin decreased drastically during holding, whereas that of ω -gliadin remained quite constant even at longer holding times (Fig. 4a). After one hour the amount of α -gliadins strongly decreased, γ -gliadins became nearly unextractable and ω -gliadin was the most important fraction in a 60% ethanol extract. The apparent

amount of reduced glutenin increased with longer holding times (Fig. 4b) reaching a maximum amount after 15 min. The total amount of extractable protein (gliadin + glutenin) lowered when holding gluten at 95 °C for 15 min or longer.

Heating to 90 °C and holding the gluten suspension at this temperature yielded results similar to those following heating and holding at 95 °C. However the viscosity rise was less pronounced (results not shown) and this was reflected in higher protein extractabilities (28.4 % SDS extractable protein after 40 min at 90 °C). Increasing the temperature to 80 °C and holding at that temperature decreased the RVA viscosity until it remained constant throughout the entire holding step (Fig. 5). Only small changes in gliadin extractability were observed (40.6 % SDS extractable gliadin after 40 min at 80 °C), although the glutenin SDS extractability (7.4 %) was strongly reduced after heating and holding at 80 °C for 40 min. However these changes were not sufficient to cause a viscosity rise in the RVA.

3.3. The effect of different amounts of gliadin and glutenin

To determine the relative contribution of the gliadin and the glutenin fraction to the overall RVA profile, gluten suspensions with different gliadin to glutenin ratios were analyzed.

The RVA profile (Fig. 6a) of glutenin enriched wheat gluten (only 17.8% gliadin on a protein basis) had a much lower initial RVA viscosity (160 cP) than the original material with 55.9% gliadin (Fig. 6c). Increasing the gliadin to glutenin ratio to the ratio in the control gluten sample resulted in a higher initial viscosity and did not markedly change the viscosity at the end of the holding phase and during cooling. Higher gliadin to glutenin ratios led to a less pronounced viscosity increase during the first 15 min of holding. Gluten with a gliadin content higher than that of the control sample (55.9%) led to a lower initial

viscosity (Table 2) and a smaller viscosity increase during holding (results not shown). This shows that both gliadin and glutenin contributed to the measured initial viscosity and the viscosity during holding at 95 °C.

The amount of gliadin (extractable in 60% ethanol) after heating and holding at 95 °C for 40 min depended on the gliadin content of the wheat gluten (Table 2). The extractability of reconstituted gluten with low and high amounts of gliadin after heating (40 min at 95 °C) was higher than that of reconstituted gluten with a gliadin amount comparable to that of unheated commercial wheat gluten (55.9%).

3.4. Determination of free SH-content during RVA analysis

Up to 70 °C, the free SH-content of wheat gluten remained constant. Between 70 °C and 80 °C a significant drop in the amount of free SH-groups occurred (Fig. 7). Simultaneously, SDS extractability of glutenin (Fig. 1) decreased, indicating cross linking of glutenin through disulphide bonding. The free SH-content then remained constant until the start of the holding step at 95 °C. This indicated a further association of glutenin through sulphydryl/disulphide interchange reactions, leading to larger protein aggregates reflected in a lower SDS extractability (Fig. 1). A second drop in free SH-content occurred after 5 min holding at 95 °C, and was accompanied by a sharp decrease in gliadin SDS extractability and a further decrease in glutenin extractability in SDS (Fig. 1). This led to the proposal that gliadin crosslinks with glutenin through formation of disulphide bonds. With longer holding times the free SH-content remained constant, although a further decrease of gliadin extractability was observed during holding at 95 °C. Addition of the SH-blocking agent (0.02 M N-ethylmaleimide) to the glutenin enriched gluten suspension

278 resulted in an RVA profile with no viscosity increase in the heating and holding phase (Fig.
279 8). This provides further evidence for the importance of thiol groups in the changes of RVA
280 viscosity when holding gluten suspensions at temperatures of at least 60 °C.

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4. Conclusions

The RVA system can be used to thermally treat wheat gluten suspensions under different conditions and to monitor changes during heating and cooling. Increasing the temperature up to 95 °C affected mainly glutenin. When holding the suspension at such temperature, both gliadin and glutenin became less extractable and the RVA viscosity increased, probably due to the formation of large protein aggregates.

A large reduction in α - and γ -gliadin extractabilities and a simultaneous increase in the apparent amounts of reduced glutenin, suggested formation of gliadin-glutenin disulphide bond cross-linking in the process. γ -Gliadins were more affected after heating than α -gliadins. The gliadins that were ethanol unextractable after heating, became extractable after reduction and eluted in the B/C-LMW-glutenin fraction.

Both the time and temperature of the holding phase affected RVA viscosity and protein extractability. Longer holding times at and above 90 °C increased the RVA viscosity. At the same time the extractability of gliadin, mainly α - and γ -gliadin and to a lesser extent ω -gliadin, and glutenin decreased. Holding at 80 °C did not increase the RVA viscosity, although glutenin extractability decreased.

Gliadin and glutenin were both responsible for the initial viscosity in the RVA profile. The formation of glutenin polymers with the incorporation of gliadin through disulphide bonds caused a viscosity rise in the RVA profile.

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312 **Fig.s**

313 Fig. 1

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Fig. 4

(a)

(b)

Fig. 5

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Fig. 7

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Fig. captions

Fig. 1. Typical RVA profile of a gluten-water suspension (20% w/v) with indication of extraction yields with 2% SDS at different times. (—) RVA viscosity, (—) temperature, (●) total SDS extractable protein, (■) SDS extractable gliadin and (▲) SDS extractable glutenin.

Fig. 2. Areas in RP-HPLC chromatogram representing gluten extractability with 60 % ethanol and 0.05 M Tris/HCl buffer (pH 7.5) with 50% propan-1-ol, 2.0 M urea and 1% (w/v) DTT after heating and cooling in the RVA at different temperatures, including room temperature (RT). Fig. 2a shows the gliadin fraction with ω -gliadin (grey), α -gliadin (black) and γ -gliadin (white). Fig. 2b shows the reduced glutenin fraction with the apparent amounts of D-LMW-GS (grey), HMW-GS (white) and B/C-LMW-GS (black); (5', holding time; C, cooling).

Fig. 3. RVA viscosity of wheat gluten-water suspension with 60 min holding time (HT) at 95 °C. (—) RVA viscosity, (—) temperature.

Fig. 4. Areas in RP-HPLC chromatogram representing gluten extractability at different HT at 95 °C in the RVA. Fig. 4a shows the gliadin fraction with ω -gliadin (grey), α -gliadin (black) and γ -gliadin (white). Fig. 4b shows the reduced glutenin fraction with of D-LMW-GS (grey), HMW-GS (white) and B/C-LMW-GS (black). 0 min HT represents extractability at the start of the holding phase.

Fig. 5. RVA viscosity of wheat gluten-water suspension with 40 min HT at 80 °C. (—) RVA viscosity, (—) temperature.

370 Fig. 6. RVA profiles (40 min HT at 95 °C) of reconstituted gluten fractions with (a) 17.8%
371 gliadin, (b) 38.6% gliadin and (c) a control gluten fraction with 55.9 % gliadin. (—) RVA
372 viscosity, (—) temperature.

373 Fig. 7. Changes in free SH-content during RVA analysis (60 min at 95 °C) as determined
374 by the reaction with DTNB in 2% (w/v) SDS, 3.0 M urea, 1.0 mM EDTA, 0.05 M
375 NaH_2PO_3 .

376 Fig. 8. RVA viscosity of wheat gluten-water suspension with 40 min HT at 95 °C. A:
377 Control; B: In 0.02 M N-ethylmaleimide. (—) RVA viscosity, (—) temperature.

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379 **Tables**

380 Table 1. 2% SDS extractability, calculated from SE-HPLC, of gluten proteins with HT up
381 to 60 min at 95 °C. Standard deviations are given between brackets.

Holding time	SDS	SDS	SDS
at 95 °C (min)	extractable	extractable	extractable
	protein (%)	gliadin (%)	glutenin (%)
Start holding	64.6 (1.5)	47.4 (1.0)	17.1 (0.5)
5	38.8 (0.9)	34.0 (0.5)	4.7 (0.4)
10	30.3 (1.0)	26.9 (0.8)	3.3 (0.2)
15	25.7 (2.1)	22.7 (1.8)	3.0 (0.2)
20	24.5 (1.0)	21.6 (0.9)	2.9 (0.1)
40	17.1 (0.0)	14.7 (0.0)	2.4 (0.0)
60	15.1 (0.1)	12.6 (0.1)	2.5 (0.1)

382

383 Table 2. Ethanol extractability of wheat gluten with a different gliadin content before and
 384 after heat treatment (40 min at 95 °C) with indication of the initial RVA viscosity of the
 385 gluten suspension. Standard deviations are given between brackets.

60% ethanol extractable gliadin before RVA treatment (%)	60% ethanol extractable gliadin after RVA treatment (40 min at 95 °C) (%)	Proportion of gliadins (%) extractable after heating	Initial RVA viscosity (cP)
17.8 (0.3)	6.0 (0.5)	33.8	160
38.6 (0.1)	11.8 (0.1)	30.6	1182
57.3 (1.0)	15.5 (0.1)	27.1	1576
61.4 (0.7)	24.1 (0.0)	39.3	1250
100	56.8 (1.3)	56.8	588

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